Phage Display

George P. Smith* and Valery A. Petrenko

Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211

Received January 2, 1997 (Revised Manuscript Received January 23, 1997)

Contents

I. *In Vitro* Evolution of Chemicals

William Paley wrote the following 57 years before the *Origin of Species*:

> In crossing a heath, suppose...I had found a *watch* upon the ground, and it should be inquired how the watch happened to be in that place....[T]he inference, we think, is inevitable, that the watch must have had a maker: that there must have existed...an artificer or artificers, who formed it for the purpose which we find it actually to answer; who comprehended its construction and designed its use...[And] every indication of contrivance, every manifestation of design, which existed in the watch, exists in the works of nature; with the difference, on the side of nature, of being greater or more, and that in a degree which exceeds all computation."[1]

But ever since Darwin we have come to understand that the exquisite "watches" of the living world are fashioned by an altogether different process. As Richard Dawkins writes in his compelling book on evolution, natural selection "does not plan for the future. It has no vision, no foresight, no sight at all. If it can be said to play the role of watchmaker in nature, it is the *blind* watchmaker."[2]

Imagine, then, the applied chemist, not as designer of molecules with a particular purpose, but rather as custodian of a highly diverse population of chemicals evolving *in vitro* as if they were organisms subject to natural selection. A chemical's "fitness" in this artificial biosphere would be imposed by the custodian for his or her own ends. For instance, the population might be culled periodically of individuals who fail to bind tightly to some biological receptor; the population would then evolve toward specific ligands for that receptor. (In this review, we will use "receptor" as a generic term for a biomolecule that specifically binds a natural ligand. This definition encompasses enzymes, which bind their substrates; hormone receptors, which bind their hormones; antibodies, which bind their antigens; and other examples.) Progress toward the custodian's chosen goal would in a sense be "automatic": once appropriate selection conditions are devised, no plan for how the system is to meet the demands of selection need be specified. And if the chemical population is sufficiently diverse, perhaps this "blind" process will outperform rational design. The custodian may not comprehend, even in retrospect, how the products of selection work, just as biologists have only the sketchiest understanding of how a fruitfly functions.

The key characteristics of evolving organisms are replicability (i.e., ability to make copies of themselves) and mutability (i.e., ability to undergo changes that are passed on to their progeny). How can a chemical "replicate" or "mutate"? Actually, the living world abounds in just such evolving chemicals. Take a protein as an example: it cannot replicate or mutate directly, of course; but it is associated with a cell or multicellular organism that can. Linkage of the protein with the genetic machinery that encodes it thus confers on it the key properties of replicability and mutability.

Phage display, the subject of this review, is a practical realization of the artificial chemical evolution envisioned above. Using standard recombinant DNA technology, peptides (or proteins; we shall often use the term "peptide" to refer to an amino acid chain regardless of its length) are associated with replicat-

S0009-2665(96)00065-9 CCC: $28.00

George P. Smith was born in Norwalk, Connecticut, in 1941. He received his A.B. degree in biology from Haverford College in 1963, and after a year as a high-school teacher and laboratory technician, went on to earn a Ph.D. in bacteriology and immunology from Harvard University in 1970. After a postdoctoral fellowship with Oliver Smithies at the University of Wisconsin, he joined the faculty of the Division of Biological Sciences at the University of Missouri in Columbia in 1975 as Assistant Professor and since has been promoted to Associate and Full Professor. He has published 35 papers in areas including antibody structure and evolution, the evolution of repeated DNA sequences, genomics, and the biology of the filamentous phage. It is from his interest in the filamentous phage that the idea of phage display evolved.

Valery A. Petrenko was born in Kalinin, USSR (now Tver, Russia) in 1949. He received B.Sc./M.Sc. degrees in chemistry in 1972 from Moscow State University, a Ph.D. in bioorganic chemistry from the Zelinski Institute of Organic Chemistry (Moscow) in 1976, and a D.Sc. in bioorganic chemistry and molecular biology from Moscow State University in 1988. He was appointed Junior Research Scientist at the Institute of Organic Chemistry in 1975. In 1977 he moved to Novosibirsk as a Senior Scientist. In 1982 he was promoted to Head of the Laboratory of Nucleic Acids, in 1985 to Assistant Scientific Director, in 1989 to Director of Institute and Assistant Scientific General Director in the Scientific Association "Vector" (now State Research Center for Virology and Biotechnology "Vector"). In 1992 he became Professor in Molecular Biology. Eight Ph.D students defended their dissertations under his supervision. He joined the faculty of the Division of Biological Sciences, University of Missouri—Columbia in 1993 and was promoted to Research Professor in 1995. His research interests include vaccine and drug development by means of phage display and by engineering of genes, proteins, and microorganisms. He has published over 70 papers and has 13 inventions and patents.

ing viral DNAs that include the peptides' coding sequences. The peptide populations so created are managed by simple microbiological methods. Phage display is an exponentially growing research area, and numerous reviews covering different aspects of it have been published in recent years.[3-18]

This review is addressed primarily to chemists, but it does assume a rudimentary knowledge of molecular biology, including replication of DNA, expression of genes (transcription of DNA into mRNA starting at a promoter, and translation of mRNA into protein using the genetic code), and use of recombinant DNA vectors to clone foreign DNA inserts.

II. Phage-Display Libraries as Populations of Replicable, Mutable Chemicals

A. Phage-Display Vectors

Phages are viruses that infect bacterial cells, and many of the vectors used in recombinant DNA research are phages that infect the standard recombinant DNA host: the bacterium *Escherichia coli*. The key feature of recombinant DNA vectors, including phages, is that they accommodate segments of "foreign" DNA—pieces of human DNA, for instance, or even stretches of chemically synthesized DNA. As vector DNA replicates in its *E. coli* host, then, the foreign "insert" replicates along with it as a sort of passenger.

An "expression vector," including a phage-display vector, has an additional feature compared to vectors in general: the foreign DNA is "expressed" as a protein. That is, it programs machinery of the *E. coli* host cell to synthesize a foreign peptide whose amino acid sequence is determined (*via* the genetic code) by the nucleotide sequence of the insert. Phage display differs from conventional expression systems, however, in that the foreign gene sequence is spliced into the gene for one of the phage coat proteins, so that the foreign amino acid sequence is genetically fused to the endogenous amino acids of the coat protein to make a hybrid "fusion" protein. The hybrid coat protein is incorporated into phage particles ("virions") as they are released from the cell, so that the foreign peptide or protein domain is displayed on the outer surface.

A phage-display "library" is a heterogeneous mixture of such phage clones, each carrying a different foreign DNA insert and therefore displaying a different peptide on its surface. Different types of libraries will be discussed below. Each peptide in the library can replicate, since when the phage to which it is attached infects a fresh bacterial host cell, it multiplies to produce a huge crop of identical progeny phages displaying the same peptide. And if the phage's DNA suffers a mutation in the peptide coding sequence, that mutation is passed on to the phage's progeny and can affect the structure of the peptide. In short, the peptides in a phage-display library have the two key characteristics required for chemical evolution: replicability and mutability.

Because of its accessibility to solvent, a displayed peptide often behaves essentially as it would if it were not attached to the virion surface. Thus, for example, peptides that are ligands for receptors typically retain their affinity and specificity when displayed in this way on the virion surface. This means that many techniques that chemists or biochemists apply to compounds free in solution can be applied more or less unaltered to peptides tethered to a phage. In particular, affinity purification, in which an immobilized receptor is used to specifically "capture" ligands from a complex mixture of compounds, can equally be used to capture phage displaying receptor-

binding peptides from a large phage library displaying many different peptide structures. The captured phages are "amplified" by infecting them *en masse* into fresh cells and culturing the cells to yield a large crop of progeny phages, which can serve as the input for another round of affinity purification. Moreover, by periodically introducing mutations into the phage population, the experimenter widens the search for effective ligands by exploring peptide sequences that are not present in the initial phage-display library (section V). Eventually, captured phages are cloned so that the displayed peptides responsible for binding can be studied individually. The amino acid sequence of the peptide is easily obtained by determining the corresponding coding sequence in the viral DNA. This so-called "affinity selection" is the premier example of artificial selection imposed on populations of phage-displayed peptides (section IV.B). Since there is no need to process clones one by one until the final stage, enormous libraries displaying billions of different structures can be easily surveyed for exceedingly rare binding clones.

B. How Foreign Peptides Are Displayed on Filamentous Phages

Most phage-display work—and all the work to be reviewed here—has used filamentous phage strains M13, fd, and f1 as the vectors; display systems based on bacteriophage T4[19,20] and λ[21] are extremely promising, but will not be reviewed here. Filamentous phages are flexible rods about 1 μm long and 6 nm in diameter, composed mainly (87% by mass) of a tube of helically arranged molecules of the 50-residue major coat protein pVIII[22]; there are 2700 copies in wild-type virions, encoded by a single phage gene VIII. Inside this tube lies the single-stranded viral DNA (ssDNA; 6407−8 nucleotides in wild-type strains). At one tip of the particle there are five copies each of the minor coat proteins pIII and pVI (genes III and VI, respectively); minor coat proteins pVII and pIX (genes VII and IX) are at the other tip. The phages infect strains of *E. coli* that display a threadlike appendage called the F pilus. Infection is initiated by attachment of the N-terminal domain of pIII (about 200 amino acids) to the tip of the pilus; this is the end of the particle that enters the cell first. As the process continues, the coat proteins dissolve into the surface envelope of the cell and the uncoated ssDNA concomitantly enters the cytoplasm. There, a complementary DNA strand is synthesized by host machinery, resulting in a double-stranded replicative form (RF). The RF replicates to make progeny RFs and is also the template for transcription of phage genes and synthesis of progeny ssDNAs. These progeny ssDNAs are extruded through the cell envelope, acquiring the coat proteins from the membrane and emerging as completed virions (several hundred per cell per division cycle). Progeny virions are secreted continuously without killing the host; chronically infected cells continue to divide, though at a slower rate than uninfected cells. The yield of virions can exceed 0.3 mg/mL.

Foreign peptides have been fused to three coat proteins: pIII, pVIII, and pVI. The first two of these are synthesized with N-terminal signal peptides,

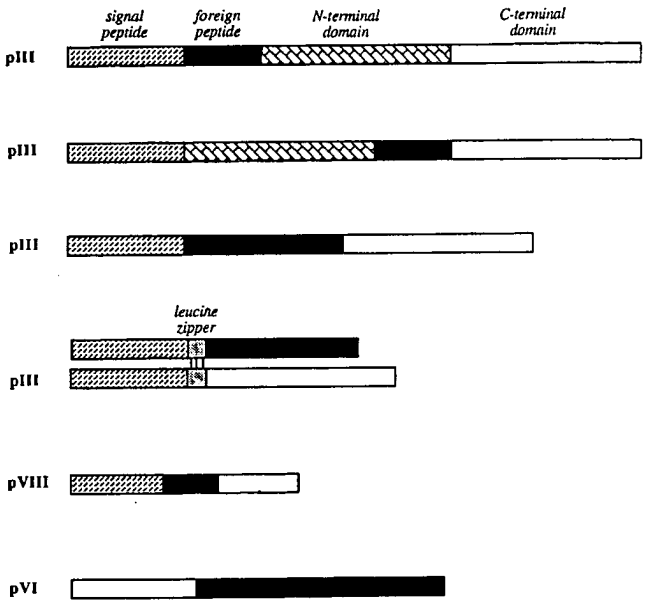

Figure 1. Schematic diagram of how foreign peptide domains are fused to coat proteins pIII, pVII, and pVI in phage-display vectors. In each diagram, the black segment represents the foreign peptide.

which are cleaved from the polypeptide chain as it is inserted in the inner membrane of the cell (the bacterial envelope has inner and outer membranes separated by the periplasm). A single segment of amino acids in pIII and pVIII spans the inner membrane, separating a periplasmic N-terminal segment from a short cytoplasmic C-terminal segment; it is from this state that the proteins are incorporated into virions.

Figure 1 diagrams the ways that foreign peptides have been fused to these proteins. Until recently, foreign peptides have been fused to regions of pVIII and pIII that were known to be exposed to the exterior: the N-terminus of pVIII[23] and the N-terminus and middle of pIII.[24,25] In some pIII vectors, the foreign peptide replaces the N-terminal domain of pIII (the third diagram in Figure 1), yielding a hybrid protein that can be incorporated into the virion but must be supplemented by complete pIII molecules if the virion is to be infective (see type 3+3 systems in the next subsection); infective virions in this case are thus mosaics with two types of pIII molecule. Similarly, when pVIII displays a relatively large foreign peptide (more than about eight amino acids), it will not support phage production unless it is supplemented by wild-type pVIII molecules, again yielding mosaic particles.[26-29]

In pIII and pVIII fusions, the foreign peptide must be spliced somewhere between the signal peptide and the portion of the coat protein that is required for incorporation into the virion. This means that the reading frame of the foreign DNA insert must be fused correctly to the reading frame of the coat protein at both vector−insert and insert−vector junctions (corresponding to the left and right ends of the black foreign peptide in Figure 1). More recently, however, foreign peptides have been fused to the C-terminus of pVI[30], as shown in the sixth diagram in Figure 1; in this case, the two reading frames need only be fused correctly at the vector−

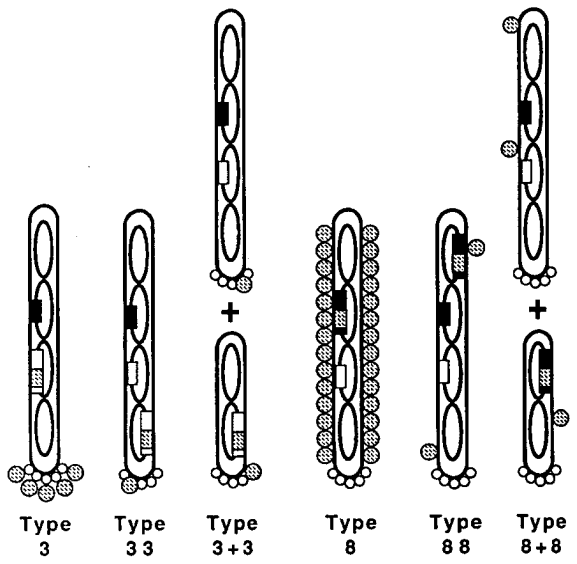

Figure 2. Types of phage display systems. Type 6, 66, and 6+6 systems are not shown. The long vertical ovals represent phage virions, and the shorter vertical ovals represent phagemid virions. The twisted line inside each virion represents the single-stranded viral DNA, the segments encoding coat proteins pVIII and pIII being designated by black and white boxes, respectively. The hatched segments within these boxes represent foreign coding sequences spliced into a coat-protein gene, and the hatched circles on the surface of the virions represent the foreign peptides specified by these foreign coding sequences. The five white circles at one tip of the virions represent the N-terminal domains of the five pIII molecules; foreign peptides displayed on pIII are either appended to the N-terminal domain (type 3 systems) or replace the N-terminal domain (type 3+3 and most type 33 systems). In type 8 systems, the foreign peptide is displayed on all copies of the major coat protein pVIII (2700 copies in wild-type virions), whereas in type 88 and 8+8 systems, only a minority of the pVIII copies display the foreign peptide. (Reproduced from ref 33. Copyright 1993 Elsevier.)

insert junction (corresponding to the left end of the black segment in Figure 1). C-Terminally fused peptides have also been displayed indirectly on pIII *via* a leucine zipper "fastener",[31] as shown in the fifth diagram. Here, both the foreign peptide and pIII are preceeded by signal peptides; after the signal peptide is removed, the two "half-zippers" join together in the periplasm or prior to, or concomitantly with, incorporation into the secreted virion.

C. Types of Phage-Display Systems

Phage-display systems can be classified according to the arrangement of the coat protein genes.[32,33] This is illustrated for fusions to pVIII and pIII in Figure 2, in which gene VIII is represented as a black block, gene III as a white block, the foreign DNA insert as a cross-hatched block, and the foreign peptide as a cross-hatched circle. In a "type 3" vector, there is a single phage chromosome (genome) bearing a single gene III which accepts foreign DNA inserts and encodes a single type of pIII molecule. The foreign peptide encoded by the insert is theoretically displayed on all five pIII molecules on a virion (though in practice normal proteolytic enzymes in the host bacterium often remove the foreign peptide from some or even most copies of pIII, especially if the foreign peptide is large). Similarly, type 8 (see Figure 2) and type 6 vectors (not shown) display foreign peptides on every copy of pVIII and pVI, respectively (no type 6 vectors have been reported). As mentioned above, only short foreign peptides can be displayed on every copy of pVIII; even so, the peptide comprises a substantial fraction of the virion's mass and can dramatically alter its physical and biological properties.[29,34,35]

In a type 88 vector, the phage genome bears two genes VIII, encoding two different types of pVIII molecule; one is ordinarily recombinant (i.e., bears a foreign DNA insert) and the other wild-type. The resulting virion is a mosaic, its coat comprised of both wild-type and recombinant pVIII molecules (the former usually predominating). This allows hybrid pVIII proteins with quite large foreign peptides to be displayed on the virion surface, even though the hybrid protein by itself cannot support phage assembly. Similarly, a type 33 vector bears two genes III, one of which is recombinant.

A type 8+8 system differs from a type 88 system in that the two genes VIII are on separate genomes. The wild-type version is on a phage (usually called the "helper" phage), while the recombinant version is on a special kind of plasmid called a "phagemid."[36,37] Like other plasmids used in recombinant DNA research, a phagemid carries a plasmid replication origin that allows it to replicate normally in an *E. coli* host and an antibiotic resistance gene that allows plasmid-bearing host cells to be selected. But it also carries a filamentous phage replication origin, which is inactive until the cell is infected with the helper phage. Then the phage replication protein acts not only on the phage origin on the helper phage DNA but also on the phage origin on the phagemid DNA. Two types of progeny virions are thus secreted: particles carrying helper phage DNA and particles carrying phagemid DNA. Both these virions, like the type 88 virions, are mosaics, whose coats are composed of a mixture of recombinant and wild-type pVIII molecules. When a phagemid virion infects a cell, the cell acquires the antibiotic resistance carried by the phagemid. When a helper phage virion infects a cell, the cell goes on to produce progeny helper virions in the normal way; the progeny virions, unlike the original infecting virion, are not mosaic, since the helper carries only a single gene VIII. Type 3+3 and 6+6 systems are like type 8+8 systems, except that the phagemid carries an insert-bearing recombinant gene III or VI, respectively, rather than VIII. The recombinant pIII encoded by a type 3+3 phagemid is usually missing the N-terminal domain (as in the third diagram in Figure 1), since cells expressing this domain are resistant to superinfection by helper phage.

Most phage display vectors are designed to be introduced into *E. coli* cells as naked DNA by electroporation,[38] which is particularly well-suited to making very large libraries. Special display vectors that can be packaged *in vitro* into phage λ particles have also been reported.[39,40]

III. Types of Displayed Peptides and Proteins

The most common type of phage-display constructs are "random" peptide libraries, an outgrowth of the

Table 1. Random Peptide Libraries

random peptide	type	N-terminal sequence[a]	no. of clones	refs
6-mer	3	ADGAX$_6$GAAG-AETVE	2×10^8	142
15-mer	3	AEX$_{15}$PPPPPP-AETVE	2×10^7	161
6-mer	3	X$_6$GG-TVE	3×10^8	141
9-mer	8+8	AEG-EFX$_9$-DPAK	4×10^7	26
10-mer	3	ADVAX$_{10}$AASG-AETVE	4×10^8	162
6-mer	3	X$_6$GGG-AETVE	nr	61
6-mer	3	AE-CX$_6$CGG-TVE	nr	61
9-mer	3	AE-LGGGGX$_9$GGGGVP-	2.4×10^7	163
15-mer	3	ADGAX$_{15}$GAAG-AETVE	4×10^7	147
6-mer	3	A-EGXCX$_4$CXSYIEGRIV-ETVE	8.6×10^6	164
9-mer	8+8	AEG-EFCX9CG-DPAK	2.5×10^7	105
36-mer	3	S(S/R)X$_{18}$PGX$_{18}$SRPAR-TVE	2×10^8	165
8-mer	3	X$_8$ASGSA-	1.4×10^9	62
12-mer	3	X$_{12}$ASGSA-	5×10^8	62
5-mer	3+3	GPGGX$_5$GGPG-	5×10^6	86
5-mer	3+3	GPAAX$_5$AAPG-	2×10^6	86
20-mer	3	ADGAX$_{20}$GAAG-AETVE	1.5×10^8	166
10-mer	3	ADASSGAX$_{10}$SALSGSG-AETVE	2×10^6	167, 168
15-mer	3	nr	5×10^7	104
7-mer	3	ADGACX$_7$CGAAG-AETVE	4.5×10^9	109
6-mer	3	X$_6$PNDKYEPFPPPPAA-AE	1×10^7	53
6-mer	3	AE-$\overline{\text{GX}_6\text{G-TVE}}$	2.5×10^9	137
6-mer	3	AE-X$_6$PPIPG-TVE	2.0×10^9	137
6-mer	3	AE-RSLRPLX$_6$G-TVE	5.8×10^8	137
6-mer	3	AE-PPPYPPX$_6$-TVE	3.1×10^8	137
6-mer	3	YGGFLGACLEPYTACDSSGGSGX$_6$[b]	2×10^8	88
5-mer	3	AE-X$_5$RPLPPLPPP-TVE	7.5×10^7	100
5-mer	3	AE-RSLRPLPPLPX$_5$-TVE	5.4×10^7	100
5-mer	3	AE-GAAPPLPPRX$_5$-TVE	2.2×10^7	100
5-mer	8+8	AEG-DDPYKCPECGKSFSQKX$_2$LX$_2$HQXTHTG-DDPA	9.7×10^6	115
6-mer	3	nr	8.6×10^6	110
6-mer	3	nr	8.6×10^8	169
15-mer	3	nr	5.7×10^8	169
9-mer	nr	nr	1×10^9	59
4-mer/Cys	3	AE-CX$_4$CIEGRGG-	3.8×10^8	170
5-mer/Cys	3	AE-CX$_5$CIEGRGG-	2.4×10^8	170
6-mer/Cys		AE-CX$_6$CIEGRGG-	6.1×10^8	170
10-mer	3	X$_{10}$GG-TVE	2×10^8	111
18-mer	3	X$_9$GAX$_9$GAAGGAGAGAG-TVE	4×10^8	111
8−12-mer/Cys	3	X$_2$CX$_{4-8}$CX$_2$GAAGGAGAGAG-TVE	3×10^8	111
30-mer	nr	nr	1×10^9	127
20-mer	88	AX$_{10}$HX$_{10}$GGSE-AEGD	1×10^9	45
(3+6)-mer	33	$\overline{(1-37)}$-X$_3$-$\overline{(41-59)}$-X$_6$-$\overline{(66-74)}$-[b]	1×10^8	116
(5+4)-mer	3	$\overline{(1-18)}$-X$_5$-$\overline{(24-80)}$-X$_4$-$\overline{(85-106)}$-[b]	2×10^8	114
6-mer	3+3	$\overline{\text{AAQPAMA-(1-7)}}$-X$_3$$\overline{\text{FX}_3\text{-(15-61)}}$[b]	8×10^7	99
35-mer	3	S(S/R)X$_{20}$(Y/H/N/D)A(I/M/T/N/K/S/R) X$_{15}$SRIEGRARPSR-[b]	5×10^8	
40-mer	3	S(S/R)$\overline{\text{X}_{20}\text{GCGX}_{20}}$SRIEGRARPSR[b]	1×10^8	57
6-mer	88	X$_6$A-AEGD	8×10^8	171
15-mer	88	X$_{15}$A-AEGD	1.3×10^9	171
30-mer	88	X$_{30}$A-AEGD	2.5×10^8	171
16-mer	88	X$_8$CX$_8$A-AEGD	2.5×10^8	171
16-mer	88	X$_{15}$CNA-AEGD	1.2×10^8	171
16-mer	88	XCX$_{15}$A-AEGD	1×10^9	171
6-mer	88	XCX$_4$CXA-AEGD	2.2×10^8	171
8-mer	88	XCX$_6$CXGGP-AEGD	1×10^{10}	171
8-mer	88	XCX$_6$CXA-AEGD	1.5×10^8	171
10-mer	88	XCX$_8$CXA-AEGD	1.5×10^9	171
9-mer	88	αct-XCCX$_3$CX$_5$C-αct[b]	5×10^8	171
8-mer	8	$\overline{\text{A-X}_8\text{-DPAK}}$	1.5×10^9	29

[a] Foreign sequences are set off by hyphens. nr = not reported. X - any amino acid. [b] Underlined residues represent the thrombin receptor tether region,[53] epitope for monoclonal antibody used for separation of phage resistant to proteases,[88] tandemistat scaffold,[116] cytochrome b_{562} scaffold,[114] minibody scaffold,[99] factor Xa protease cleavage site,[57] and α-conotoxin scaffold.[171]

synthetic "mimotope" strategy of Geysen and his coworkers;[41,42] such libraries are listed in Table 1. In this case, the DNA inserts are derived from "degenerate" oligonucleotides, which are synthesized chemically by adding mixtures of nucleotides (rather than single nucleotides) to a growing nucleotide chain. In the degenerate sequence NNKNNKNNKNNK, for example, each N is an equal mixture of A, G, C, and T; each K is an equal mixture of G and T; each NNK is a mixture of 32 triplets that include codons for all 20 natural amino acids; and the entire 12-base sequence is an equimolar mixture of over a million (32^4) different molecular species collectively encoding all 160 000 (20^4) possible 4-residue peptides. Degeneracy at the level of whole codons, rather than single nucleotides, can give a less biased representation of

Table 2. Proteins Displayed on Filamentous Phages

protein	type	refs
genomic libraries		
DNA *Staphylococcus aureus*	3+3, 8+8	172,173
cDNA libraries		
Aspergillus fumigatus	3+3[a]	174,175
Ancylostoma caninum	6	30
fragments of proteins		
β-galactosidase	3	25
bluetongue virus capsid protein VP5	3	136
plasminogen-activator inhibitor 1	3+3	176
RNA polymerase II	3+3	132
enzymes		
alkaline phosphatase	3	177
	8+8	178
	3+3[a]	31
	3+3	179
trypsin	3+3, 8+8, 33, 88	90,180
prostate specific antigen	3+3	181
β-lactamase	3	85, 182−184
cytochrome b_{562}		
glutathione transferase	3+3	185
staphylococcal nuclease	3	186
	3+3	187
lysozyme	3+3	179
hormones		
human growth hormone	3+3	112, 188
atrial natriuretic peptide	3+3	189
	3+3	190
angiotensin	33, 3+3	32
endothelin	33	116
inhibitors		
bovine pancreatic trypsin inhibitor	88	130
	3	58
	3	96
plasminogen activator inhibitor	3+3	191,192
Kunitz domain of Alzheimer's amyloid β-protein precursor	3+3	64,98
cystatin	3+3	193
ecotin	3+3	89,90
Tendamistat	33	116
lipoprotein-associated coagulation inhibitor	3	68
toxins		
Aspergillus fumigatus ribotoxin	3+3[a]	31
ricin B chain	3	194
receptors		
IgE receptor (α subunit)	3+3	195
protein A (B domain)	3+3	196
IgG binding domain from group G *Streptococcus*	3	117,197,198
T cell receptor	8+8	199
CD4 domains 1 and 2	88	200
ligands		
Ligands for Src homology 3 domain	nr[b]	137
		100
Urokinase plasminogen activator fragment 13-32	3	51
thrombin receptor activating peptide	3	53
substance P	3	53
neurokinin A	3	53
neurokinin B	3	53
epitopes and antigens		
epitope of malaria parasite *Plasmodium falciparum*	8	28
	8+8	126
	3	25
chlamidial epitope	88	107
DNA and RNA binding proteins		
zinc fingers	3	153
	3+3	154, 156
	33	155, 157−159
U1A protein	3+3	201
enzyme substrates		
protease substrates	3+3	86, 87
cytokines		
interleukin 3	3	202
ciliary neurotrophic factor	3+3	43, 203
interleukin-6	3+3	204
cDNA libraries		
Aspergillus fumigatus	3+3[a]	174,175
Ancylostoma caninum	6+6	30

Table 2 (Continued)

protein	type	refs
fragments of proteins		
β-galactosidase	3	25
VP5 of bluetongue virus	3	136
plasminogen-activator inhibitor 1	3+3	176
RNA polymerase II	3+3	132

[a] Displayed through a leucine zipper fastener (section II.B). [b] nr = not reported.

the amino acids in the random peptides.[43-45] A typical random peptide library has about a billion phage clones—enough to represent most of the 64 million possible 6-mers, but far too small to represent the 3×10^{19} possible 15-mers.

Table 2 lists constructs that display all or part of natural peptide or protein domains, rather than random peptides. In "genomic" libraries, the inserts are fragments of total chromosomal DNA; thus, all coding sequences in the organism's genetic complement (i.e., "genome") are potentially represented among the displayed peptides. Similarly, in cDNA libraries the inserts are DNA copies of messenger RNAs (mRNAs) extracted from some tissue or cell population; again, a huge diversity of coding sequences is potentially represented in a sufficiently large cDNA library. In the remaining constructs in Table 2, the phages display all or part of a specific peptide or protein domain. In many cases, some positions in the displayed domain are "randomized" in some way to create a library of sequence variants, usually with an eye to selecting rare clones with enhanced function, or clones in which the displayed domain has acquired a new function as a result of mutation.

IV. Selection

A. General Principles

Selection consists of culling an initial population of phage-borne peptides to give a subpopulation with increased "fitness" according to some user-defined criterion. In most cases, the input to the first round of selection is a very large initial library (10^9 clones, each represented by 100 particles on average, are typical numbers) and the selected subpopulation is a tiny fraction of the initial population (10^6 particles, say), fitter clones being overrepresented. This population can be "amplified" by infecting fresh bacterial host cells, so that each individual phage in the subpopulation is represented by millions of copies in the amplified stock. The amplified population can then be subjected to further rounds of selection (perhaps accompanied by mutagenesis) to obtain an ever-fitter subset of the starting peptides.

There are two pivotal parameters of selection, which can often be manipulated to some extent in order to enhance the efficacy of selection. *Stringency* is the degree to which peptides with higher fitness are favored over peptides with lower fitness; *yield* is the fraction of particles with a given fitness that survive selection. The ultimate goal of selection is usually to isolate peptides with high fitness, but this does not mean that stringency should be increased without bound, since increasing stringency usually entails decreased yield. High yield of the fittest clones is of paramount importance in the very first round of selection, whose input consists of all clones in a very large initial library. Using the typical numbers in the previous paragraph, suppose that each clone in the library—including the very fittest clones that are the desired end-product of selection—is represented by only 100 particles on average. If the yield for the fittest clones is not greater than 1%, such clones have a good chance of being lost and of course can never be recovered. Those clones that do survive the first round of selection are amplified and are thus represented by millions of phages each in subsequent rounds; yield can then safely be decreased in favor of high stringency. There is a limit to stringency, however, because in practice selection techniques are imperfect and there is an unavoidable background yield of all phages regardless of their fitness. If stringency is set too high, the yield of a specifically selected phage will fall far below the background of a nonspecifically isolated phage, and all power of discrimination in favor of high fitness is lost.

B. Affinity Selection

As mentioned already in section II.A, by far the most common selection pressure imposed on phage-displayed peptide populations is affinity for a target receptor. Affinity selection is ordinarily accomplished by minor modifications of standard affinity purification techniques in common use in biochemistry. Thus the receptor is tethered to a solid support, and the phage mixture is passed over the immobilized receptor. Those phages—usually a tiny minority—whose displayed peptides bind the receptor are captured on the surface or matrix, allowing unbound phages to be washed away. Finally, the bound phages are eluted in a solution that loosens receptor–peptide bonds, yielding an "eluate" population of phages that is greatly enriched (often a million fold or more) for receptor-binding clones. The eluted phages are still infective and are propagated simply by infecting fresh bacterial host cells, yielding an "amplified" eluate that can serve as input to another round of affinity selection. Phage clones from the final eluate (typically after 2–3 rounds of selection) are propagated and characterized individually. The amino acid sequences of the peptides responsible for binding the target receptor are determined simply by ascertaining the corresponding coding sequence in the viral DNA. In general, high stringency is favored by low densities of the target receptor[46] and by monovalent display of the foreign peptide;[47] high stringency is almost invariably accompanied by relatively low yield. In the remainder of this subsection, we will review ways in which these general principles have been implemented.

The solid supports to which target receptors are tethered can be classified into surface supports—polystyrene dishes,[24] impermeable plastic beads,[47] nitrocellulose membranes,[48] and paramagnetic beads[49]—and permeable beaded agarose gels.[50] Permeable agarose beads are convenient to use and have a very high capacity per unit volume. However, it seems unlikely that phage particles, whose long dimension (~1 μm) is orders of magnitude larger than the average diameter of the pores of an agarose gel, can diffuse far into the interior of a bead; for this reason, only receptors tethered at the very surface of a bed may actually be effective at capturing phage.

Receptors can be directly attached to the solid support by chemical coupling[47,50] or noncovalent adsorption to a hydrophobic plastic surface.[24] Alternatively, receptor molecules can be biotinylated and allowed to bind to a surface that has already been coated with avidin or streptavidin, thereby attaching them indirectly through the superstrong biotin–avidin or biotin–streptavidin bond.[25] There are numerous other ways of indirectly attaching the receptor to the solid support; indeed whole cells in suspension or attached to a culture dish can be used as a solid support to select ligands for cell-surface receptors.[51-54]

Indirect attachment *via* a biotin moiety allows a "two-step" mode of capture:[25] in the first step, the phage mixture is reacted with biotinylated receptor in homogeneous solution; in the second, the mixture is reacted with streptavidin-coated solid support in order to capture those phages whose displayed peptide bound the biotinylated receptor during the first step. In principle, at least, two-step capture allows the kinetics of the binding reaction to be controlled without the complications attendant on surface reactions.

After the capture step (whether part of a one- or two-step procedure), the solid support is washed to remove unbound phages and eluted under conditions that release the bound phages without impairing their infectivity. Nonspecific elution conditions are intended to weaken receptor–peptide interactions without regard to their specificity. They exploit the high resistance of filamentous phage to denaturation by acidic buffers with pH's down to 2.2,[24] alkaline buffers such as 0.1 M triethylamine,[55] urea concentrations as high as 6 M at pH 2.2 (G.P.S., unpublished), and proteases such as trypsin[56] and factor Xa.[57] Gradients of acidity[58] or other agents[59] have been used in an attempt to elute phages in order of increasing affinity. This approach should be used with caution, however, since in most cases it is not clear *a priori* how closely the affinities of receptor–peptide bonds correlate with their resistance to denaturing conditions.

Specific elution seeks to release phages that are bound to the target receptor's binding site, without releasing phages that are bound for some other reason—for example, by interaction with a contaminant, or with the carrier protein that is often used to block nonspecific adsorption sites on the solid support after the target receptor itself has been immobilized. In competitive elution, a known soluble ligand for the receptor competes with phage for binding to immoblilized receptor.[60-64] This is a two-stage process: the phage-borne peptides must first dissociate spontaneously from the solid support, then the competitor binds the receptor binding site thus freed, reducing its availability for rebinding phage-borne peptide. Thus if the time course of dissociation is long on the scale of the experiment, competitive elution will fail. Noncompetitive elution, in contrast, relies on a compound that specifically loosens binding by the receptor without binding to its binding site, and without weakening binding interactions in general. For instance, phage bound to a calcium-dependent receptor can be eluted with the calcium chelator EGTA;[65,66] this greatly increases the specificity of elution, since only rarely would a nonspecifically bound phage happen to be held in a calcium-dependent fashion.

It is actually not necessary to elute the captured phage at all. Simply adding fresh bacterial host cells to the solid support allows the captured phage to infect cells and thus be propagated.[67] The yield is generally low (1–10% of the yield from elution; G.P.S., unpublished), but in all but the first round of selection is probably sufficient to ensure retention of binding clones (see above). So far, this "elution by infection" has been reported only for peptides displayed on pVIII; it is not clear how well it will work if the peptide is displayed on pIII.

In some cases, the unamplified eluate is directly subjected to another round of selection.[68] Because the yields of even the highest-affinity clones at each round of selection seldom approach 100%, however, overall yields decline sharply with successive rounds; so it is important to start with an initial population in which each clone is represented by sufficient numbers of particles to guard against extinction if it happens to be a good binder. Also, some elution conditions seem to somehow physically alter the phages (without impairing infectivity) so that the background yield in the next round is much higher than with amplified phages.[25]

Several groups[69-71] have introduced a promising variant of affinity selection that does not rely on physical capture on a solid support. Here, the peptide is displayed on a mutant version of coat protein pIII that is missing its N-terminal domain, as in the third line of Figure 1. Since this domain is required for infectivity, these particles are noninfective. Infectivity can be restored by attaching the missing N-terminal domain to a receptor that binds the phage-borne peptide. Therefore, only phage displaying peptides that bind the receptor are infective and are thus amplified. This sets up a sort of "automatic" evolution in which an initially highly diverse population of peptides evolves toward higher affinity for the receptor as the phage grow in the presence of host bacteria. There are many variations on this theme. For instance, if each phage clone in the initial library encodes both a randomized peptide on the defective pIII and a randomized receptor fused to the N-terminal domain, this system can be used to isolate peptide–receptor pairs with affinity for each other.[71]

Because selected phage are in the end cloned and characterized one by one, it is feasible to use a

complex mixture of receptors, rather than a single receptor, to capture phage. For instance, total human serum immunoglobulin, comprising hundreds or thousands of individual antibody specificities, can be used to select a diversity of peptides, each recognized by one of the specificities. This is the basis of "epitope discovery",[72-83] a strategy for identifying diagnostic peptides and synthetic vaccine components (section VIII.E).

The progress of affinity selection through succeeding rounds is ordinarily reflected in increasing affinity of individual phage clones or of entire eluate populations for the target receptor. The affinity of individual clones or entire eluate populations can be assessed quantitatively by standard enzyme-linked immunosorbent assay (ELISA).[14] Alternatively, a few hundred individual clones from an eluate can be sampled on "plaque lifts" and tested qualitatively for ability to bind the receptor.[72]

C. Selection for Traits Other than Affinity

In principle, at least, phage-borne peptides might be selected on the basis of fitness criteria other than affinity for a target receptor. Thus, for example, Petrenko and co-workers selected phage clones from a type 8 library that are resistant to extraction with chloroform.[29] In practice, however, almost all selection procedures have involved affinity at least indirectly. Suicide inhibitors[84] are a case in point, as illustrated in experiments of Soumillion and colleagues.[85] They incubated a library of phage displaying variants of β-lactamase with a special β-lactam substrate coupled to biotin. This substrate is converted by β-lactamase to a highly reactive form that couples itself to the β-lactamase enzyme. Thus phage displaying catalytically active β-lactamase molecules become marked with a biotin moiety and can be specifically captured out of a vast mixture of unmodified phage by their high affinity for immobilized streptavidin.

Affinity has similarly been used indirectly to select for protease substrates.[86-90] In these projects, a peptide or protein domain with high affinity for a convenient receptor is fused to pIII coat protein through a randomized amino acid sequence. The phage library is bound to a solid support coated with the receptor and then exposed to the protease. Those phages whose randomized amino acid sequence happens to be a substrate for the protease are released from the solid support and can be propagated by infecting fresh cells. By sequencing the randomized peptide's coding sequence within the viral DNA in these phage clones, amino acid sequences that are effective substrates for the protease can be ascertained.

To select peptides that home to the brain, Pasqualini and Ruoslahti[91] injected phage libraries into the tail vein of mice, recovered phages from brain or kidney a few minutes later, amplified the phages by infecting fresh bacterial host cells, and reinjected to initiate the next round of selection. Peptides capable of mediating selective localization of phage to brain and kidney blood vessels were identified in this way and showed up to 13-fold selectivity for these organs. It is likely that specific homing is based on affinity for a saturable tissue receptor of some sort, since it could be blocked by simultaneous administration of the free peptide.

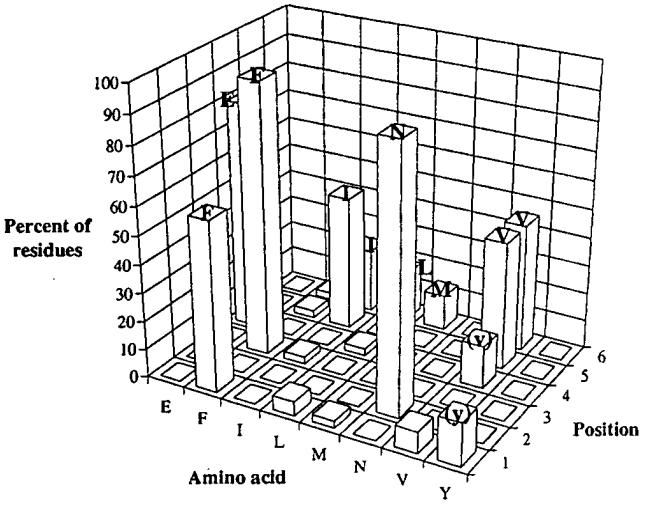

Figure 3. Distributions of amino acids observed at six randomized positions in phages affinity-selected with ribonuclease S-protein. Amino acids classified as motif residues are labeled with letters; they all have frequencies of at least 13% at the indicated position, whereas nonmotif residues have frequencies no greater than 8%. A motif residue whose frequency is no greater than 25% of the frequency of the next most abundant amino acid at that position is classified as a minor motif residue and labeled with a lower-case letter enclosed in parentheses; the remaining motif residues are classified as major and are labeled with upper-case letters.

D. Enrichment of Specific Sequence Motifs

Increasing fitness is typically accompanied by emergence of a common "motif" in the amino acid sequences of the selected peptides (sometimes more than one motif). For instance, Smith and co-workers[92] (also D. A. Schultz, J. E. Ladbury, G.P.S. and R. O. Fox, unpublished) used ribonuclease S-protein to affinity-select peptides from a library displaying 50 million random hexapeptides. The incidence of the amino acids at each of the six randomized positions after three rounds of selection is graphed in Figure 3. It is evident that the sequence FNFE greatly predominates at positions 1−4 and that just a few chemically similar amino acids dominate at positions 5 and 6 as well (V/I and V/I/L/M, respectively). Twelve of the 20 natural amino acids did not appear at any position in any of the clones, though they were present at roughly the expected frequencies in the initial library. Overall, the 6-mer motif (F/y)NF(E/v)(V/I)(V/I/L/M) is evident, where lower-case letters indicate minor motif residues (defined in the legend) and amino acids enclosed in parentheses are alternatives observed at a given position.

It is noteworthy that this motif does not resemble any part of the amino acid sequence of S-protein's natural ligand, S-peptide; there is no way it could have been predicted by rational design, despite 35 years of intensive work on the S-protein/S-peptide system. Similarly, Wrighton and colleagues[93] isolated a small peptide that is a full agonist of the much larger erythropoietin hormone, but shares no significant similarity with it at the amino acid sequence

level. In general, emergence of entirely unexpected motifs is a recurring theme in the results of selections from random peptide libraries and testimony to the power of selection to reveal bioactive structures that could not be discovered by rational design.

V. Exploring the Fitness Landscape

A. Sequence Space, Fitness Landscapes, and Sparse Libraries

The ensemble of all possible combinations of amino acids at randomized positions in a library (e.g., of all 64 million possible hexapeptides for a random 6-mer library) comprise an abstract geometric domain that is commonly called "sequence space"; each individual sequence is thus a point in this sequence space. In this section, we will represent sequence space as a two-dimensional plane and individual sequences as points on that plane. We must not take the analogy to a map too literally, however: there is no clear relationship between the distance separating two sequences in any geometric representation—even a highly abstract one with multiple dimensions—and the resemblance of those sequences' physical and chemical properties. Still, it will be useful in what follows to suppress this complication and consider points that are close in our two-dimensional representation to represent similar amino acid sequences. More specifically, the two axes of the plane could represent the possible sequences in nonoverlapping subsets of the randomized positions which we will call regions 1 and 2; region 1 could be positions 1−4 of a random octapeptide, for example, and region 2 positions 5−8.

Imagine, then, adding one more dimension—a third axis in our grossly simplified but heuristically useful representation. On this axis we plot the fitness of each of the sequences in sequence space according to the selection criterion being imposed on the peptide population. These closely spaced points form a surface overlying sequence space. Parts A and B Figure 4 illustrate two hypothetical "fitness landscapes" overlying two-dimensional sequence space. The researcher's goal, in these terms, can be understood as searching through sequence space in order to find the highest point he or she can on the fitness landscape—the sequence that is fittest by the artificially imposed selection criterion. In no practical case are the staggering amounts of data required to plot an actual empirical fitness landscape available; nevertheless, thinking about possible fitness landscapes is a mental device that can help in devising more efficient search strategies.

The fitness landscapes depicted in Figure 4A,B differ in two important ways. First, the peak in Figure 4A is broad and smooth, whereas Figure 4B has multiple peaks, including a sharp one, that give it a more "rugged" character. Second, the landscape in Figure 4A has a special kind of symmetry. If we choose an arbitrary point in region 2, and keeping that sequence fixed plot fitness over the one-dimensional sequence space of region 1, we obtain a curve (Figure 4C) that we will call a "transect" through the fixed point in region 2. This transect has the same relative shape (though very different absolute heights), regardless of which region 2 sequence it passes through. Similarly, all perpendicular transects through fixed points in region 1 have the same shape (in this case, a broader shape than in Figure 4C). This symmetry implies that the fitness at each point in sequence space can be written as the product of a fitness contributed by region 1 and a fitness contributed by region 2. Equivalently, taking the logarithm of both sides of this equation, we arrive at an alternative definition of fitness to which regions 1 and 2 make additive contributions. If, to give a concrete example, fitness consists of affinity for a target receptor, the symmetry illustrated in Figure 4A means that overall affinity is the product of affinities contributed by regions 1 and 2 separately. And since the free energy of binding is proportional to the logarithm of affinity, this also means that the overall free energy of binding is the sum of free energies of binding contributed separately by regions 1 and 2. When such symmetry is present, we say that regions 1 and 2 make "independent" or "additive" contributions to fitness, and correspondingly we will call the fitness landscape itself additive. It is obvious that the landscape in Figure 4B is far from additive: a transect through a point in region 2 that lies close to the broad peak (transect A in Figure 4D) is completely different from a transect lying closer to the sharp peak (transect B in Figure 4D). Whether or not a fitness landscape is additive in this sense depends on how the random positions are parsed into regions. For instance, if the random residues occur in two separate loops in a protein domain, the sequences in the two loops might well make independent contributions to fitness, whereas a subdivision that groups residues from the two loops into a single region might result in a markedly nonadditive landscape.

A phage-display library can be seen as a collection of random points in sequence space. Only for the tiniest sequence spaces do these points represent all or most possible sequences. For example, there are 3×10^{19} possible random 15-mers, whereas the largest phage-display libraries comprise only about 10^{10} individual clones—an exceedingly sparse sampling on the scale of the relevant sequence space.

In principle, at least, selection can identify the fittest clone(s) in the library—the peptide(s) corresponding to the highest point(s) on the fitness landscape; we will call such a clone an "initial champion." Because of the sparseness of the library, however, an initial champion's fitness may be far inferior to that of the globally fittest clone(s) in sequence space. This is especially likely if maximum fitness lies atop a sharp peak in a rugged landscape (Figure 4B)—a narrow topographical feature that may be missed altogether in a sparse sampling. The more clones in the library, and the less biased the representation of the different amino acids,[43−45] the less severe this deficit is likely to be, underscoring the desirability of large libraries. Once constructed, a large, general-purpose library is an extremely valuable resource, since it can be replicated indefinitely by infecting fresh bacterial host cells and widely distributed for use in an unlimited number of projects.

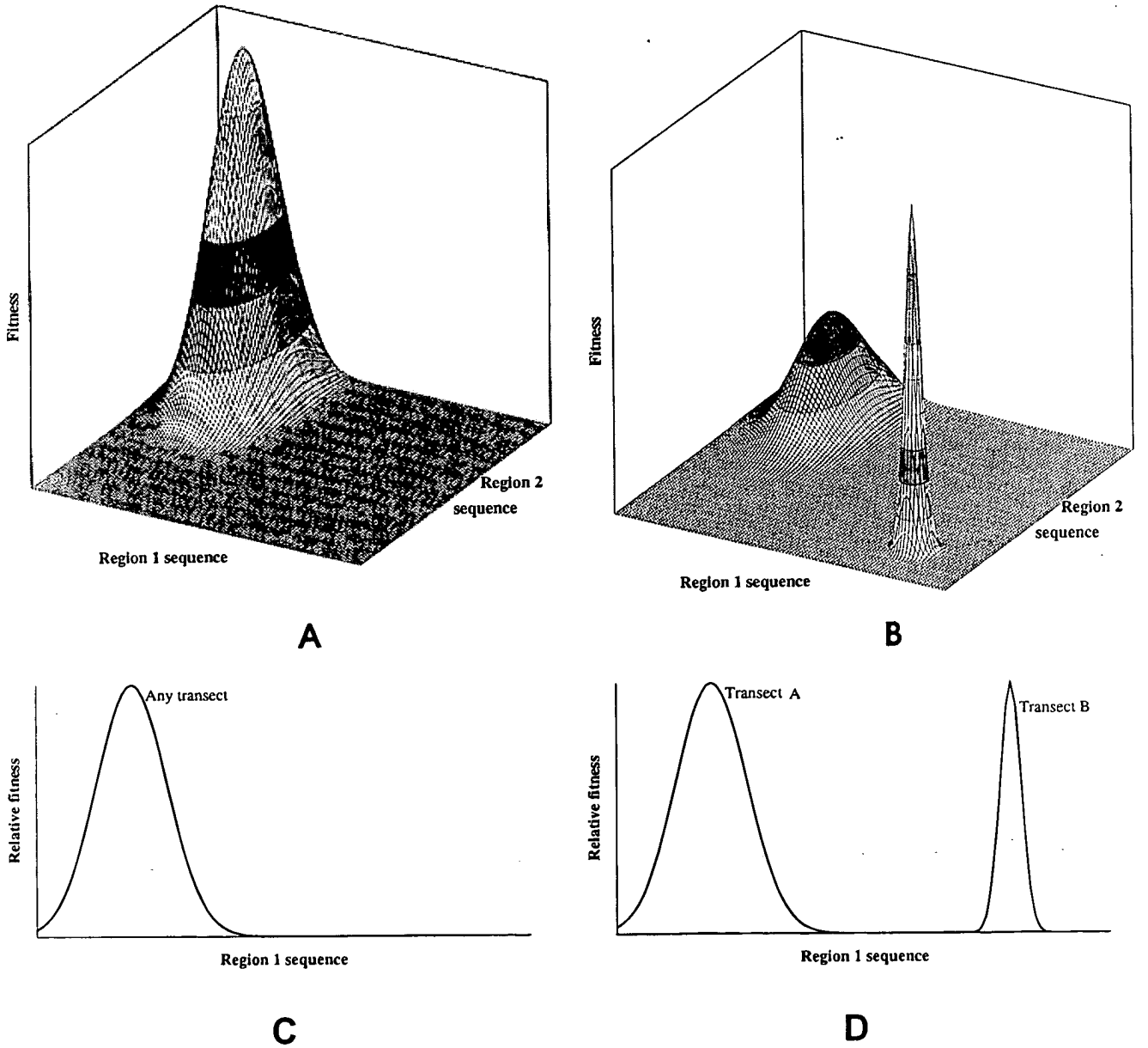

Figure 4. Fitness landscapes: (A, top left) a nonrugged, additive landscape; (B, top right) a rugged, nonadditive landscape; (C, bottom left) a transect of the additive landscape in A through any sequence in region 2 (all transects have identical shapes); (D, bottom right) two transects of the nonadditive landscape in B, passing through two sequences in region 2 (different transects can have markedly different shapes).

B. Strategies for Exploring the Fitness Landscape

A researcher who is not content with the fitness of the initial champion obtained from one library might of course try new libraries. But that is a woefully inefficient way of accessing additional points in sequence space, even leaving out of consideration the fact that construction of a new library is an arduous affair. Natural evolution suggests a much better strategy: introduce random mutations into a population that has already been subjected to selection, then select again from the resulting mutagenized population to obtain even fitter clones. In effect, the search through sequence space is concentrated in the close neighborhoods of clones that, having survived a previous round of selection, are enriched for sequences with at least some level of fitness.

The first round of selection favors clones at higher elevations in the fitness landscape and disfavors clones at lower elevations. The survivors are then propagated and mutagenized (mutagenesis methods will be discussed at the end of this section), each thus giving rise to a "clan" of variant descendants. Assuming mutagenesis is moderate, these descendants will lie close to their progenitor in sequence space—some at higher elevation, some at lower. In the next round of selection, usually at higher stringency, fitter variants are again selected over less-fit ones. As the population experiences each successive cycle of mutagenesis and ever more stringent selection, more and more clans become extinct, while the remaining ones "climb" toward the tops of their local fitness peaks.

The "greedy" strategy is one implementation of this program. First, the initial library is subjected to multiple rounds of increasingly stringent selection in the hope of selecting the very best clone—the initial champion.[43,94] This single clone is then mutagenized

to generate a single clan of variants, from which yet fitter clones are selected. And so on, until further rounds of stringent selection and mutagenesis yield no further improvement in fitness—i.e., until the summit of the local fitness peak is achieved.

The greedy strategy is inherently risky, however, because the initial champion may lie on a local fitness peak of much lower elevation than the globally highest peak. Consider, for instance, a rugged fitness landscape like that in Figure 4B, where the highest peak has a very small footprint in sequence space and thus will be sampled very infrequently in the initial library, while a much lower peak has a much larger footprint and thus will be sampled frequently. It is highly plausible, in such a case, that the initial champion will happen to lie on a broad, low feature and that far superior sequences lie at completely different locations in sequence space—locations that are too far distant from the initial champion to be accessible by mutagenesis. Of course, the researcher will not know—certainly not in advance—that the fitness landscape is like this, but it may be prudent to design a search strategy that has a chance of overcoming this limitation. An attractive "non-greedy" approach is to relax the stringency of selection in the first cycle, so that not only the initial champion but also many clones of inferior (but still above-average) fitness survive.[95] This larger subpopulation has a chance of including "dark horses": clones that are inferior to the initial champion, but that lie near higher fitness peaks. This entire subpopulation is then mutagenized *en masse* to generate a new library, which is subject to the next round of selection. This scenario resembles natural evolution much more closely than does the greedy search.

Even if there is a dark horse in the initial library, there is no guarantee that its descendants will ultimately win the competition, since the descendants of the initial champion start with a competitive advantage; nor can we point to any case where a dark horse has been actually demonstrated empircally. Furthermore, there is a disadvantage to the non-greedy approach: because limited search resources must be distributed among many neighborhoods in sequence space (one for each clone in the selected subpopulation), it is not possible to search the neighborhood of the initial champion nearly as thoroughly as in the greedy method. Perhaps, if the stakes are high enough, both approaches are worth trying.

The "stepwise" or "iterative" strategy is another approach to searching sequence space.[64,96-100] Here, the randomized positions are divided into two or more subsets or regions, like regions 1 and 2 in Figure 4A,B. The sequence in one of the regions—region 2, say—is fixed, while the other region (region 1) is randomized; in terms introduced in the previous subsection, this is equivalent to thoroughly exploring a single transect of sequence space passing through a single point in region 2 (Figure 4C,D). The possible sequences in region 1 will be much more thoroughly represented in this restricted library than in a library in which both regions 1 and 2 are randomized simultaneously. The fittest clone in the transect is then selected from the restricted library, to identify an optimal region 1 sequence. The process is then reiterated, but this time the region 1 sequence is fixed at its optimum, region 2 is thoroughly randomized, and again the fittest clone is selected. In theory, the optimum region 1 and region 2 sequences identified in this two-step process should together constitute the overall optimum sequence. This supposition is justified for an additive fitness landscape like Figure 4A, since every transect yields the same optimum (Figure 4C); but for nonadditive landscapes like Figure 4B, the results can be very different, depending on what happens to be chosen as the fixed sequence in region 2 (Figure 4D). A stepwise search makes sense when the randomized positions can be subdivided into well-defined, separate parts of a protein domain.

Two main methods have been used to introduce mutations into selected clones. Error-prone polymerase chain reaction (PCR) is used when many clones must be simultaneously mutagenized *en masse*, as in the nongreedy strategy.[101-103] In contrast, when random mutations must be introduced into a confined segment of a single clone, as is typically the case in the greedy and step-wise strategies, incorporation of degenerate oligonucleotides (as in the contruction of random peptide libraries; section II.C) is an efficient method in which the frequency and uniformity of mutations can be easily controlled.

VI. Effect of Conformational Constraints

Unlike natural proteins or protein domains, random peptides do not generally fold into a well-defined three-dimensional structure. However, constraints can be artificially imposed on the peptide in order to greatly reduce the range of conformations available to it. In general, a library of constrained peptides will represent far fewer three-dimensional shapes than a library of unconstrained (but otherwise comparable) peptides. As a consequence, the probability that a clone will posess the target activity—affinity for a receptor, for instance—is correspondingly reduced.[74,104] On the other hand, a constrained peptide whose accessible conformations happen to overlap extensively with active conformations may possess far higher activity than any unconstrained peptide.

The most common constraint on displayed peptides is a disulfide bond between two half-cystine residues at fixed positions in an otherwise random sequence; many such constructs are listed in Table 1. Because the phage coat proteins are secreted into the oxidizing milieu of the periplasm and ultimately secreted into extracellular medium with abundant dissolved oxygen, cysteine residues within a single displayed peptide can be expected to form intrapeptide disulfides in at least a portion of the displayed peptides; interchain disulfides are much less likely, since the distance between neighboring coat-protein subunits is at least 10 times longer than a disulfide bond. In several cases, the disulfide bond has been shown to be required for the ability of the displayed peptide to bind a target receptor.[32,61,93,105-110] In general, the closer the half-cystines, the tighter the constraint imposed on the amino acids lying between them. Thus, disulfides spanning different numbers of amino

acid positions would be expected to impose very different, mutually exclusive conformational constraints when the numbers are small. In contrast, disulfides spanning more than about six residues probably impose relatively weak constraints that are compatible with a great diversity of conformations.

Coordination bonds between histidine residues and metal ions can constrain peptides in much the same way as disulfide bonds. De Ciechi and co-workers, for instance, reported that a monoclonal antibody affinity-selected peptides with the motif HXG(A/T)-XH and that binding was abolished in the presence of the metal-chelating agents EDTA and EGTA.[111]

A second way of constraining peptides is to present them in the context of a protein scaffold. In this case, random peptides can be presented not only as loop structures, but also as parts of α helices, β sheets, β turns, and other elements of secondary structure. Table I includes many such libraries; the host scaffolds include human growth hormone,[112] bovine pancreatic trypsin inhibitor,[96] antibodies,[113] minibodes (next paragraph), cytochrome b_{562},[114] zinc fingers,[115] Tandemistat,[116] Kunitz domain,[64,98] and the IgG-binding domain of streptococcal protein A.[117,118] With the exception of the minibody, zinc-finger, and Tandemistat, most of these scaffolds have displayed a single randomized loop or region with the aim to optimize the binding of expressed modified protein with its natural receptor. Here we will consider the applications of protein scaffolds for construction of universal constrained peptide libraries.

Minibodies have been particularly thoroughly investigated as a host scaffold.[99,119-122] A minibody is a 61-residue peptide comprising three strands from each of the two β-sheets of an immunoglobulin variable region domain, along with the exposed H1 and H2 hypervariable regions. Such a minibody was displayed on the surface of the f1 bacteriophage and the two hypervariable loops randomized to create a constrained peptide library; from this library clones were affinity-selected for high affinity to human IL-6.

Bianchi and his colleagues constructed a conformationally homogeneous peptide library by randomizing five positions in the α-helical portion of a zinc-finger motif displayed on pVIII.[115] A monoclonal antibody specific for the lipopolysaccharide of the human pathogen *Shigella flexneri* was used to affinity-select clones from the library, yielding a consensus motif with strong, zinc-dependent affinity for the antibody. Moreover, affinity for the antibody was retained when the same five side chains were transferred to a synthetic scaffold that holds them in an α-helical geometry. This ability to transfer a motif to a peptidomimetic scaffold has great potential importance for use of phage display for drug discovery, since peptides themselves are not considered auspicious starting points for therapeutics (section VII.D).

VII. Applications

A. Target Receptors Used in Affinity Selection

In Table 3 we list target receptors that have been used to affinity-select peptides from phage-display libraries. It is evident that the diversity of targets is very wide, encompassing not only conventional receptors like antibodies and hormone receptors but also (for instance) plastic surface[123] and whole organs in a living mouse.[91] Although most of the receptors recognize natural ligands that are proteins, some of them recognize nonproteinaceous ligands like carbohydrates, and some (e.g., plastic surface) have no natural ligand at all. In the subsections that follow, we will discuss a few of the major applications that these selection experiments have in mind.

B. Epitope Mapping and Mimicking

An "epitope" is the small determinant on the surface of a ligand with which the receptor makes close, geometrically and chemically specific contact. If the ligand is a protein, the epitope is sometimes "continuous," comprising a few adjacent critical amino acids in the primary sequence. For instance, antibodies specific for continuous epitopes on protein antigens typically contact three to four critical amino acids over a six-residue segment. More often, however, protein epitopes are more complex. Many are "discontinuous" because they comprise critical binding residues that are distant in the primary sequence but close in the folded native conformation. And many epitopes, including discontinuous ones, are "conformation-dependent" because they require the context of the overall protein structure to contrain them in a binding conformation.

In many research contexts, it is highly desirable to "map" the epitope to a confined portion of the natural protein ligand. If the epitope is (or might be) continuous and not conformation dependent, random peptide libraries provide a cheap, easy approach to this goal.[13,80,105,124-134] The receptor is used to affinity select random peptide ligands, and the sequence motif in the selected peptides is compared to the amino acid sequence of the natural ligand. Often, in these cases, the motif clearly matches critical binding amino acids in the natural protein ligand, thereby mapping the epitope to a very narrow part of the overall natural ligand structure. Since this approach uses replicable, widely available, all-purpose random peptide libraries and simple microbiological procedures, it is generally much cheaper and easier than alternative epitope mapping methods that require chemical synthesis of short peptide segments of the ligand's amino acid sequence.[135]

Only rarely will a random peptide library contain a binding motif extending to more than about six amino acids or adequately represent conformation-dependent or discontinuous epitopes. Although receptors recognizing such epitopes often select ligands from random peptide libraries, these artificial ligands seldom bear a recognizable similarity to any part of the natural protein ligand at the amino acid sequence level. An alternative approach in such circumstances is to construct a gene-specific library displaying 15–100 amino acid segments of the natural amino acid sequence[132,136]—long enough to occasionally include small elements of secondary structure from the native protein. Such libraries sometimes contain good ligands for receptors that fail to select ligands from random peptide libraries. Because it requires

Table 3. Target Receptors Used for Affinity-Selection Experiments[a]

receptors	library	refs
monoclonal antibody antibodies against		
tumor suppressor protein p53	pIII/X_6	133
	pIII/X_{12}, X_{20}	134
HIV gp120	pIII/X_{10}	162
	pIII/X_{15}	147
	pIII/X_{30}	127
fibroblast growth factor	pIII/X_6	144
carbohydrate Lewis^Y antigen	pIII/X_8, CX_6C	128
acetylcholine receptor	pIII/X_6	143
angiotensin II	pIII/X_8, CX_6C	32
glycoprotein D of herpes simplex virus type I	pIII/X_{15}	148
oncoprotein p185^{HER2}	pVIII/X_9, CX_9C	149
keratin	pIII/X_6, X_{12}, X_{20}	124,205
plasminogen activator inhibitor type-1	pIII/X_6	110
bluetongue virus VP7	pIII/X_6	206
FLAG octapeptide	pIII/X_{10}	168
Na^+/K^+-ATPase β-subunit	pIII/X_{15}	106
hepatitis B virus surface antigen	pIII/X_{15}	76
	pIII/X_{30}	125
dengue virus	pIII/X_6	207
dystrophin	pIII/X_{15}	208
von Willebrand factor	pIII/X_{15}	209
HCV core protein	pIII/X_{30}	127
β-Endorphin	pVIII/X_{20}	45
acetylcholine receptor	pIII/X_6	210
cytochrome *b*	pIII/X_6, X_9	211
proenkephalin	pIII/X_6, X_{12}, X_{20}	212
Cell surface antigen B7-1	pIII/X_{10}, X_{18}, $CX_{8-12}C$	111
prostate-specific membrane antigen	pIII/X_{37}, X_{43}	57
P^1,P^4-diadenosine 5′-tetraphosphate receptor	pIII/X_6	146
36-mer peptide from viral hemagglutinin, cyclic peptides, *Pseudomonas aeruginosa* pilin, Boretella pertusis pilin, HIV gp120, rabbit muscle, L-type calcium channels, worm muscle myohemerythrin, lysozyme, trisaccharide on the O-antigen of *Salmonella paratyphi*, tetrasaccharide on the O-antigen of *Shigella flexneri*	11 pVIII libraries	171
polyclonal antibodies		
anti-biotin	pIII/X_6	213
anti-human lymphotoxin		214
anti-mouse IgG Fc	pIII/X_{38}	165
from sera of rheumatoid arthritis patients	pVIII/X_9	79,83
anti-TNFα from rheumatoid arthritis sera of patients	pVIII/X_9	80
anti-hepatitis B virus envelope protein	pVIII/CX_9C	72
anti-lymphotoxin	pIII/X_6, X_{15}	214
anti-synthetic peptide	pVIII/9-mer	82
from synovial fluid of rheumatoid arthritis patients	pVIII/X_9	81
	pIII/X_6	83
from sera of Hepatitis C virus-infected patients	pVIII/X_9, CX_9C	74
chimeric antibody		
ELAM1-mouse IgG2b	X_{20}	45
nonantibody protein receptors		
streptavidin	pIII/X_{15}	161,169
	pIII/X_6	142
	pIII/X_6	213
	pIII/X_{38}	165
	pIII/CX_6C	164
	pIII/$CX_{4-6}C$	170
HLA-DR	pIII/X_9	163,215,216
	pIII/X_{15}	217
concanavalin A	pIII/X_6, X_8	60,218
calmodulin	pIII/X_{15}	65
tumor suppressor protein p53	pIII/X_6 X_{12}, X_{20}	219
Src homology 3 (SH3) domains	pIII/X_{15}	104,137
	pIII/X_6	100
	pIII/X_8, X_{22}, X_{36}	138,139
	pIII/X_{10}	145
Scr homology 3 domain (D-stereomer)	pIII/X_{10}	145
Urokinase receptor	nr	51
integrin IIb/IIIa	pIII/CX_6C	117
integrin $\alpha_5\beta_1$	pIII/X_6	108
	pIII/X_7	109
heat shock cognate protein Hsc70	pIII/X_6, X_{15}	169
tissue factor VIIa	nr	64,98
atrial natriuretic peptide A receptor	nr	189
BiP chaperone	pIII/X_8, X_{12}	62

Table 3 (Continued)

receptors	library	refs
nonantibody protein receptors		
fibronectin	pIII/CX_6C	63
erythropoeitin receptor	Various	93
E-selectin	pIII/X_8, X_{12}, $CX_{2-6}C$	94
CD1-β2M complex	pVIII/X_{22}	220
stromelysin, matrilysin	pIII/X_6	88
tissue-type plasminogen activator	pIII/X_6	221
Ca^{2+} binding protein S-100b	pIII/X_{15}	66
α-chymotrypsin	pIII/X_6	222
HIV-1 nucleocapsid protein NCp7	pVIII/CX_9C	67
core antigen of hepatitis B virus	pIII/X_6	48
fibroblast growth factor receptor 1	X_{26}	54
trypsin	pIII/X_6	223
nucleic acids		
single stranded DNA	pIII/X_6	160
matrix attachment region DNA	pIII/CX_9C	59
small organic ligands		
biotin	pVIII/CX_9C	224
dioxin	pVIII/X_8	29
whole cells		
insect and mammalian cells expressing human urokinase plasminogen activator receptor	pIII/X_{15}	51
platelets	pIII/X_{15}	52
	pIII/X_6	53
organs		
brain, kidney	pIII/X_9, $CX_{5-7}C$, $CX_{18}C$, X_{20}	91
plastic	pIII/X_{36}, X_{22}	123

[a] nr = not reported.

construction of a specific library for each new ligand gene, however, this approach is much more arduous than use of all-purpose random peptide libraries.

As emphasized in section IV.D, affinity selection from random peptide libraries often reveals entirely unexpected ligands—ligands that do not match any linear epitope and that could not have been anticipated from even extensive knowledge of the receptor and/or its natural ligand. This is especially so when the receptor's natural epitope is nonproteinaceous or is a discontinuous or conformation-dependent protein epitope (previous paragraph). Geysen and his colleagues introduced the term "mimotope" to refer to small peptides that specifically bind a receptor's binding site (and in that sense mimic the epitope on the natural ligand) without matching the natural epitope at the amino acid sequence level;[41,42] the definition includes cases where the natural ligand is nonproteinaceous. Mimotopes are usually of little value in mapping natural epitopes, but may have other important uses, as will be illustrated in the next few sections.

C. Identifying New Receptors and Natural Ligands

A ligand for a receptor can be used as a "probe" to identify new receptors that bind the same ligand. Sparks and his colleagues[138-140] and others[100,104,137] used this approach to identify novel SH3 domains—a family of homologous, ~60-residue, protein-binding modules found in a great variety of signaling and cytosceletal proteins. In the first step, a number of cloned SH3 domains were used to affinity-select specific ligands from random peptide libraries. Then, in the second step, these peptides were used to probe a conventional cDNA expression library for proteins that bind the peptide. Eighteen SH3 domains were identified in this way, nine of which were previously unknown. Systematic studies like these serve to deepen understanding of cell biology by interconnecting signaling pathways not hitherto known to be related.

In a few very favorable cases, identifying peptide ligands from a random peptide library may suffice to find the natural ligand for an "orphan receptor"—a receptor whose natural ligand is unknown. Thus, for example, Ivanenkov *et al.*[66] affinity-selected peptides that specifically bind the Ca^{2+}-dependent binding protein S-100b. They shared a motif of eight amino acids, and analysis of sequence data banks identified a similar motif in the α-subunit of actin capping proteins. The interaction of these two proteins was subsequently shown to be biologically significant.

D. Drug Discovery

Many of the receptors used in affinity selection are targets of drug discovery programs, and the peptide ligands selected by them are therefore potential leads to new drugs.[53,61,64,94,98,108,109,120,122,141-146] Such peptides might act as receptor agonists or antagonists (for example, of enzymes or hormone receptors) or otherwise modulate the receptor's biological effect.

Affinity selection resembles in essence the traditional approach to drug discovery: screening libraries of synthetic compounds or natural products for substances that bind the target receptor and that might therefore be leads to new agonists, antagonists, or modulators. There are important differences, however. Affinity selection has the key advantage that the scale of the search is many orders of magnitude greater than is feasible when chemical libraries must be screened compound by compound—billions of peptides *versus* tens of thousands of chemicals. On the other hand, for most pharmaceu-

tical applications, peptides have poor pharmacological properties, being generally orally unavailable and subject to rapid degradation in the body by naturally occurring enzymes. There is some precedent for synthesizing peptidomimetic compounds that mimic the essential pharmacological features of bioactive peptides on a nonpeptide scaffold (section VI). But developing peptidomimetics is an arduous and chancy project in medicinal chemistry, and it seems likely that the most important contribution of phage display to drug discovery will be confined to applications where peptides themselves can serve as plausible therapeutics. For example, Wrighton and colleagues[93] used phage display to identify a small peptide agonist of the receptor for eythropoietin, a protein hormone that is administered parenterally in some circumstances. The small peptide, which bears little resemble to the natural hormone at the amino acid sequence level, might serve as a superior substitute for the much larger protein. Vaccines (next subsection) are another case in which peptides are eminently usable therapeutics.

Peptides composed of D-amino acids are much less susceptible to degradation in the body than peptides composed of the natural L-amino acids. Schumacher and his colleagues have put forth a clever (if expensive!) way of using phage display to identify D-amino acid peptide ligands for target receptors.[145] They synthesized chemically the D form of an SH3 domain and used it to affinity select ligands from a random peptide library, whose amino acids are of course the natural L isomers. The D forms of these peptides are therefore ligands for the natural L form of the receptor—the form that would be the actual target of drug discovery.

E. Epitope Discovery—A New Route to Vaccines and Diagnostics

When the receptor used for affinity selection is an antibody, the peptides it selects from random peptide libraries are called "antigenic mimics" of the corresponding natural epitope—the antigenic determinant that elicited the selector antibody in the first place. When these peptides are used in turn to immunize naive animals, some are able to elicit new antibodies that cross-react with the natural epitope, even though the naive animals have never been directly exposed to it.[63,72,74,76,77,80,83,147-150] Such peptides are "immunogenic mimics" as well as antigenic mimics.

By no means are all antigenic mimics immungenic mimics in this sense,[151] however, and undoubtedly many failures of immunogenic mimicry have gone unreported. There are at least two highly plausible scenarios according to which a peptide that binds its selector antibody (thus qualifying as an antigenic mimic) would not be able to elicit cross-reacting antibodies when used to immunize naive animals (thus failing as an immunogenic mimic). First, if it is flexible—and most small peptides are—it might adopt one conformation when it binds the selector antibody but myriad other conformations when it elicits new antibodies, few if any of which would therefore cross-react with the authentic epitope. Second, a peptide may be an antigenic mimic without being a true structural mimic. Such a peptide would bind the selector antibody in an entirely different way than does the original authentic epitope, *via* altogether different interactions. Just so, peptides with the motif −HPQ− bind the biotin-binding pocket of streptavidin differently than does biotin itself.[152] Such a peptide would be expected to elicit new antibodies that fit it in an altogether different way than does the original selector antibody; only rarely and coincidentally would these antibodies cross-react with the authentic epitope.

Antigenic and immunogenic mimicry are the basis of "epitope discovery",[72-83] a new approach to disease diagnosis and vaccine development. Most diseases—particularly infectious diseases—leave their imprint on the complex mixture of antibody specificities that comprises the total serum immunoglobulin population. Included in this population are disease-specific antibodies—some elicited directly by antigens on a pathogen, others possibly recognizing antigens that reflect the disease process more indirectly. When total serum antibody from a patient is used to affinity select clones from a random peptide library, therefore, some of the selected ligands will correspond to disease-specific antibodies. Of course the patient's pool of antibodies will contain myriad non-disease-specific antibodies, too, so it may require extensive counterselection or screening with antibodies from control subjects (not suffering from the disease) to identify those peptides that correspond to authentic disease-related antibody specificities and that therefore can be considered diagnostic for the disease. This is an eminently "portable" program of discovery, using the same procedure and the same "all-purpose" random peptide libraries regardless of the particular disease. Even in the most difficult cases, it nets a rich diversity of diagnostic peptides with far less work than is required to identify antigenic peptides by direct study of a pathogen's antigenic makeup.

Peptides obtained through epitope discovery have at least two obvious uses. First, as antigenic mimics they serve as specific probes for antibodies that are diagnostic for the diseases, much as natural viral proteins serve in current tests for HIV. Their advantages over natural antigens as diagnostic reagents include that they are easier and cheaper to discover and manufacture, that they can focus on a few particularly diagnostic specificities and exclude potentially confusing signals from nondiagnostic determinants, and that they can be discovered and used even when the natural antigens associated with the disease are entirely unknown.

The second possible use of peptides obtained through epitope discovery is as components of synthetic vaccines. Only antigenic mimics that are also immunogenic mimics are useful in this regard, of course, since in order to be protective an antibody must react with a natural epitope on the actual pathogen.

F. Selection of DNA-Binding Proteins

Phage display may help molecular biologists realize a long-standing goal: to design proteins that specifically bind a given target DNA sequence. Rational design has poor prospects in this field, since there do not seem to be simple rules of comple-

mentarity—comparable to those governing base-pairing between complementary single-stranded nucleic acids—by which the sequence specificity of a DNA-binding protein can be predicted from the amino acids at criticial positions in its structure. A much more promising approach is to construct a library of randomized variants of a parent DNA binding domain (e.g., one of the zinc-finger domains, a common DNA-binding motif in eukaryotic nuclei) displayed on a filamentous phage; randomization is concentrated on positions that are thought to make sequence-specific contacts with the target DNA in the parent domain. From this library, clones that bind a new target DNA sequence, different from that recognized by the parent domain, are then affinity-selected.[153-159]

In an experiment analogous to epitope mapping (subsection B above), phage display has been used to map the DNA binding site of SATB1, a nuclear matrix protein that specifically binds the minor groove of a DNA sequence motif called MAR. Using an MAR DNA sequence as the immobilized receptor, Wang and colleagues[59] affinity-selected peptides from a random peptide library; the predominant peptide shared 50% sequence identity with a nine-residue segment of the SATB1 sequence—a segment that was subsequently shown on independent grounds to be critical for DNA recognition. Phage display has also been used to affinity-select a hexapeptide with some binding preference for the single-stranded heptadeoxycytidilate $(dC)_7$, although in this case no mapping purpose was in view.[160]

G. Landscape Libraries as a Source of New Materials

The surface landscape of a filamentous virion is a cylindirical array of thousands of repeating subunits composed of the exposed parts of the major coat protein pVIII; this exposed shell accounts for about half the weight of the particle. When a random peptide is displayed on every copy of this protein, it subtends a major fraction (20% or more) of the repeating unit and thus of the entire particle surface. Unless the random peptide is loosely tethered to the bulk of the major coat protein, it is forced to interact with residues in its immediate neighborhood, and may therefore be constrained in a definite three-dimensional conformation that differs markedly from the surface conformation of wild-type particles[35] and of clones displaying other random peptides. A large population of such clones can therefore be regarded as a library of "organic landscapes".[29]

The ensemble of a random peptide in a landscape library with its surrounding wild-type residues may have emergent properties that are lost when the peptide is excised from its context. Such peptides are analogous to the complementarity-determining regions of antibodies—oligopeptide loops that in the context of the intact protein make most of the specific contacts with antigen but as free peptides seldom have appreciable antigen-binding propensities. In most applications to date, such emergent properties inhere in a single peptide and its immediate neighborhood. Localizable emergent properties are present even when the foreign peptide is displayed on only an occasional pVIII molecule, as in type 88 and 8+8 systems. Nevertheless, the high-density display in landscape phage may greatly enhance overall effectiveness in some applications. For instance, if a single target receptor complex can bind two or more neighboring peptides on the phage surface, the overall effective affinity may be enhanced many orders of magnitude compared to monovalent binding.

Some emergent properties are not localizable to a single subunit but seem instead to be a global property of the entire surface landscape. Thus, for instance, phage clones that are highly resistant to chloroform were selected from a landscape library; their entire surface is composed of hybrid pVIII subunits displaying a peptide motif that confers resistance to the solvent. In contrast, mosaic phage coated with roughly equal numbers of such hybrid subunits and wild-type subunits showed almost no resistance, indicating that resistance is not an additive property to which each hybrid subunit contributes independently.[29]

Landscape phage might be looked on as a new kind of submicroscopic "fiber." Each phage clone is a type of fiber with unique surface properties. These fibers are not synthesized one by one with some use in mind. Instead, billions of fibers are constructed, propagated all at once in a single vessel, and portions of this enormous population are distributed to multiple end-users with many different goals. Each user must devise a method of selecting from this population those fibers that might be suitable for his or her particular application—by affinity selection or whatever other selection principle ingenuity can conjure up.

Localizable or global emergent properties cannot be transferred from the virion surface to another medium; any application that depends on such properties must therefore use phages themselves as the new material. This undoubtedly precludes some applications of phage "fibers": it is doubtful we will be wearing clothes made of them, for instance. Still, filamentous phages are essentially proteins manufactured by a fermentation process and as such are potentially usable in any of the myriad of applications that might be contemplated for such proteins.

H. Phage Display—Combinatorial Chemistry on the Cheap

For drug discovery and a handful of other high-profile applications with high commercial stakes, phage display is perhaps not an optimal technology. For the ordinary research user, however, it has the overwhelming advantage that it is cheap and easy. It uses standard microbiological techniques that are familiar to all molecular biologists, and its key resources—phage libraries and clones—are replicable and therefore nearly cost-free after their initial construction or selection. It is astonishing to contemplate that within a single 1.5-mL microcentrifuge tube we can fit a few hundred trillion phage particles displaying billions of different peptide structures—an abundance and diversity from which hundreds of different users with altogether different purposes in mind can select clones of great value. And when that tube's supply has nearly run out, we have only to

propagate what is left to satisfy the needs of hundreds of additional penurious users.

VIII. References

(1) Paley, W. *Natural Theology*; 2nd ed.; J. Vincent: Oxford, 1828.
(2) Dawkins, R. *The Blind Watchmaker*; W. W. Norton: New York, 1987.
(3) *Phage Display of Peptides and Proteins*; Kay, B. K., Winter, J., McCafferty, J., Eds.; Academic Press: New York, 1996.
(4) Ladner, R. C. *Trends Biotechnol.* **1995**, *13*, 426–430.
(5) Felici, F.; Luzzago, A.; Monaci, P.; Nicosia, A.; Sollazzo, M.; Traboni, C. *Biotechnol. Annu. Rev.* **1995**, *1*, 149–183.
(6) O'Neil, K. T.; Hoess, R. H. *Curr. Opin. Struct. Biol.* **1995**, *5*, 443–449.
(7) Cortese, R.; Monaci, P.; Nicosia, A.; Luzzago, A.; Felici, F.; Galfre, G.; Pessi, A.; Tramontano, A.; Sollazzo, M. *Curr. Opin. Biotechnol.* **1995**, *6*, 73–80.
(8) Perham, R. N.; Terry, T. D.; Willis, A. E.; Greenwood, J.; di Marzo Veronese, F.; Appella, E. *FEMS Microbiol. Rev.* **1995**, *17*, 25–31.
(9) Winter, J. *Drug Dev. Res.* **1994**, *33*, 71–89.
(10) Scott, J. K.; Craig, L. *Curr. Opin. Biotechnol.* **1994**, *5*, 40–48.
(11) Makowski, L. *Curr. Opin. Struct. Biol.* **1994**, *4*, 225–230.
(12) Barbas, S. M.; Barbas, C. F. *Fibrinolysis* **1994**, *8*, 245–252.
(13) Clackson, T.; Wells, J. A. *Trends Biotechnol.* **1994**, *12*, 173–184.
(14) Lane, D. P.; Stephen, C. W. *Curr. Opin. Immunol.* **1993**, *5*, 268–271.
(15) Hoess, R. H. *Curr. Opin. Struct. Biol.* **1993**, *3*, 572–579.
(16) Scott, J. K. *Trends Biochem. Sci.* **1992**, *17*, 241–245.
(17) Cesareni, G. *FEBS Lett.* **1992**, *307*, 66–70.
(18) Smith, G. P. *Curr. Opin. Biotechnol.* **1991**, *2*, 668–673.
(19) Efimov, V. P.; Nepluev, I. V.; Mesyanzhinov, V. V. *Virus Genes* **1995**, *10*, 173–177.
(20) Ren, Z. J.; Lewis, G. K.; Wingfield, P. T.; Locke, E. G.; Steven, A. C.; Black, L. W. *Protein Sci.* **1996**, *5*, 1833–1843.
(21) Sternberg, N.; Hoess, R. H. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 1609–1613.
(22) Marvin, D. A.; Hale, R. D.; Nave, C.; Citterich, M. H. *J. Mol. Biol.* **1994**, *235*, 260–286.
(23) Ilyichev, A. A.; Minenkova, O. O.; Tatkov, S. I.; Karpyshev, N. N.; Eroshkin, A. M.; Petrenko, V. A.; Sandakhchiev, L. S. *Dokl. Biochem. (Proc. Acad. Sci. USSR)—Engl. Transl.* **1989**, *307*, 196–198.
(24) Smith, G. P. *Science* **1985**, *228*, 1315–1317.
(25) Parmley, S. F.; Smith, G. P. *Gene* **1988**, *73*, 305–318.
(26) Felici, F.; Castagnoli, L.; Musacchio, A.; Jappelli, R.; Cesareni, G. *J. Mol. Biol.* **1991**, *222*, 301–310.
(27) Iannolo, G.; Minenkova, O.; Petruzzelli, R.; Cesareni, G. *J. Mol. Biol.* **1995**, *248*, 835–844.
(28) Greenwood, J.; Willis, A. E.; Perham, R. N. *J. Mol. Biol.* **1991**, *220*, 821–827.
(29) Petrenko, V. A.; Smith, G. P.; Gong, X.; Quinn, T. *Protein Eng.* **1996**, *9*, 797–801.
(30) Jespers, L. S.; Messens, J. H.; De Keyser, A.; Eeckhout, D.; Van Den Brande, I.; Gansemans, Y. G.; Lauwereys, M. J.; Vlasuk, G. P.; Stanssens, P. E. *Biotechnology* **1995**, *13*, 378–382.
(31) Crameri, R.; Suter, M. *Gene* **1993**, *137*, 69–75.
(32) McConnell, S. J.; Kendall, M. L.; Reilly, T. M.; Hoess, R. H. *Gene* **1994**, *151*, 115–8.
(33) Smith, G. P. *Gene* **1993**, *128*, 1–2.
(34) Kishchenko, G. P.; Minenkova, O. O.; Ilyichev, A. A.; Gruzdev, A. D.; Petrenko, V. A. *Mol. Biol.-Engl. Transl.* **1991**, *25*, 1171–1176.
(35) Kishchenko, G.; Batliwala, H.; Makowski, L. *J. Mol. Biol.* **1994**, *241*, 208–213.
(36) Mead, D. A.; Kemper, B. In *Vectors: A survey of molecular cloning vectors and their uses*; Rodriquez, R. L., Denhardt, D. T., Eds.; Butterworths: Boston, 1988.
(37) Cesareni, G. In *Vectors: A survey of molecular cloning vectors and their uses*; Rodriquez, R. L., Denhardt, D. T., Eds.; Butterworths: Boston, 1988.
(38) Dower, W. J.; Miller, J. F.; Ragsdale, C. W. *Nucleic Acids Res.* **1988**, *16*, 6127–6145.
(39) Hogrefe, H. H.; Amberg, J. R.; Hay, B. N.; Sorge, J. A.; Shopes, B. *Gene* **1993**, *137*, 85–91.
(40) Alting-Mees, M. A.; Short, J. M. *Gene* **1993**, *137*, 93–100.
(41) Geysen, H. M.; Rodda, S. J.; Mason, T. J. *Mol. Immunol.* **1986**, *23*, 709–715.
(42) Geysen, H. M.; Rodda, S. J.; Mason, T. J. *Ciba Found. Symp.* **1986**, *119*, 130–149.
(43) Saggio, I.; Gloaguen, I.; Poiana, G.; Laufer, R. *EMBO J.* **1995**, *14*, 3045–3054.
(44) Glaser, S. M.; Yelton, D. E.; Huse, W. D. *J. Immunol.* **1992**, *149*, 3903–3913.
(45) Haaparanta, T.; Huse, W. D. *Mol. Diversity* **1995**, *1*, 39–52.
(46) Barret, R. W.; Cwirla, S. E.; Ackerman, M. S.; Olson, A. M.; Peters, E. A.; Dower, W. J. *Anal. Biochem.* **1992**, *204*, 357–364.
(47) Bass, S.; Greene, R.; Wells, J. A. *Proteins* **1990**, *8*, 309–314.
(48) Dyson, M. R.; Murray, K. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2194–2198.
(49) Fowlkes, D. M.; Adams, M. D.; Fowler, V. A.; Kay, B. K. *Biotechniques* **1992**, *13*, 422–428.
(50) McCafferty, J.; Griffiths, A. D.; Winter, G.; Chiswell, D. J. *Nature* **1990**, *348*, 552–554.
(51) Goodson, R. J.; Doyle, M. V.; Kaufman, S. E.; Rosenberg, S. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 7129–7133.
(52) Fong, S.; Doyle, L. V.; Devlin, J. J.; Doyle, M. V. *Drug Dev. Res.* **1994**, *33*, 64–70.
(53) Doorbar, J.; Winter, G. *J. Mol. Biol.* **1994**, *244*, 361–369.
(54) Doyle, M. V.; Doyle, L. V.; Fong, S.; Goodson, R. J.; Panganiban, L.; Drummond, R.; Winter, J.; Rosenberg, S. In *Combinatorial Libraries. Synthesis, Screening and Application Potential*; Cortese, R., Ed.; Walter de Gruyter: Berlin–New York, 1996.
(55) Harrison, J. L.; Williams, S. C.; Winter, G.; Nissim, A. *Methods Enzymol.* **1996**, *267*, 83–109.
(56) Salivar, W. O.; Tzagoloff, H.; Pratt, D. *Virology* **1964**, *24*, 359–371.
(57) McConnell, S. J.; Uveges, A. J.; Fowlkes, D. M.; Spinella, D. G. *Mol. Diversity* **1996**, *1*, 165–176.
(58) Roberts, B. L.; Markland, W.; Siranosian, K.; Saxena, M. J.; Guterman, S. K.; Ladner, R. C. *Gene* **1992**, *121*, 9–15.
(59) Wang, B.; Dickinson, L. A.; Koivunen, E.; Ruoslahti, E.; Kohwi-Shigematsu, T. *J. Biol. Chem.* **1995**, *270*, 23239–23242.
(60) Oldenburg, K. R.; Loganathan, D.; Goldstein, I. J.; Schultz, P. G.; Gallop, M. A. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5393–5397.
(61) O'Neil, K. T.; Hoess, R. H.; Jackson, S. A.; Ramachandran, N. S.; Mousa, S. A.; DeGrado, W. F. *Proteins* **1992**, *14*, 509–515.
(62) Blond-Elguindi, S.; Cwirla, S. E.; Dower, W. J.; Lipshutz, R. J.; Sprang, S. R.; Sambrook, J. F.; Gething, M. J. *Cell* **1993**, *75*, 717–728.
(63) Pasqualini, R.; Koivunen, E.; Ruoslahti, E. *J. Cell Biol.* **1995**, *130*, 1189–1196.
(64) Dennis, M. S.; Lazarus, R. A. *J. Biol. Chem.* **1994**, *269*, 22137–22144.
(65) Dedman, J. R.; Kaetzel, M. A.; Chan, H. C.; Nelson, D. J.; Jamieson, G. A., Jr. *J. Biol. Chem.* **1993**, *268*, 23025–23030.
(66) Ivanenkov, V. V.; Jamieson, G. A., Jr.; Gruenstein, E.; Dimlich, R. V. *J. Biol. Chem.* **1995**, *270*, 14651–14658.
(67) Lener, D.; Benarous, R.; Calogero, R. A. *FEBS Lett.* **1995**, *361*, 85–88.
(68) Markland, W.; Roberts, B. L.; Ladner, R. C. *Methods Enzymol.* **1996**, *267*, 28–51.
(69) Gramatikoff, K.; Georgiev, O.; Schaffner, W. *Nucleic Acids Res.* **1994**, *22*, 5761–5762.
(70) Duenas, M.; Borrebaeck, C. A. K. *Biotechnology* **1994**, *12*, 999–1002.
(71) Krebber, C.; Spada, S.; Desplancq, D.; Pluckthun, A. *FEBS Lett.* **1995**, *377*, 227–231.
(72) Folgori, A.; Tafi, R.; Meola, A.; Felici, F.; Galfre, G.; Cortese, R.; Monaci, P.; Nicosia, A. *EMBO J.* **1994**, *13*, 2236–2243.
(73) Cortese, R.; Felici, F.; Galfre, G.; Luzzago, A.; Monaci, P.; Nicosia, A. *Trends Biotechnol.* **1994**, *12*, 262–267.
(74) Prezzi, C.; Nuzzo, M.; Meola, A.; Delmastro, P.; Galfre, G.; Cortese, R.; Nicosia, A.; Monaci, P. *J. Immunol.* **1996**, *156*, 4504–4513.
(75) Mennuni, C.; Santini, C.; Dotta, F.; Farilla, L.; Di Mario, U.; Fierabracci, A.; Bottazzo, G.; Cortese, R.; Luzzago, A. *J. Autoimmunity* **1996**, *9*, 431–436.
(76) Motti, C.; Nuzzo, M.; Meola, A.; Galfre, G.; Felici, F.; Cortese, R.; Nicosia, A.; Monaci, P. *Gene* **1994**, *146*, 191–198.
(77) Meola, A.; Delmastro, P.; Monaci, P.; Luzzago, A.; Nicosia, A.; Felici, F.; Cortese, R.; Galfre, G. *J. Immunol.* **1995**, *154*, 3162–3172.
(78) Felici, F.; Galfre, G.; Luzzago, A.; Monaci, P.; Nicosia, A.; Cortese, R. *Methods Enzymol.* **1996**, *267*, 116–129.
(79) Dybwad, A.; Forre, O.; Kjeldsen-Kragh, J.; Natvig, J. B.; Sioud, M. *Eur. J. Immunol.* **1993**, *23*, 3189–3193.
(80) Sioud, M.; Dybwad, A.; Jespersen, L.; Suleyman, S.; Natvig, J. B.; Forre, O. *Clin. Exp. Immunol.* **1994**, *98*, 520–525.
(81) Dybwad, A.; Forre, O.; Natvig, J. B.; Sioud, M. *Clin. Immunol. Immunopathol.* **1995**, *75*, 45–50.
(82) Dybwad, A.; Bogen, B.; Natvig, J. B.; Forre, O.; Sioud, M. *Clin. Experimental Immunol.* **1995**, *102*, 438–442.
(83) Sioud, M.; Forre, O.; Dybwad, A. *Clin. Immunol. Immunopathol.* **1996**, *79*, 105–114.
(84) Ator, M. A.; de Montellano, O. In *The Enzymes*; 3rd ed.; Academic Press:, 1990; Vol. 19.
(85) Soumillion, P.; Jespers, L.; Bouchet, M.; Marchand-Brynaert, J.; Winter, G.; Fastrez, J. *J. Mol. Biol.* **1994**, *237*, 415–422.
(86) Matthews, D. J.; Wells, J. A. *Science* **1993**, *260*, 1113–1117.
(87) Matthews, D. J.; Goodman, L. J.; Gorman, C. M.; Wells, J. A. *Protein Sci.* **1994**, *3*, 1197–1205.
(88) Smith, M. M.; Shi, L.; Navre, M. *J. Biol. Chem.* **1995**, *270*, 6440–6449.
(89) Wang, C. I.; Yang, Q.; Craik, C. S. *J. Biol. Chem.* **1995**, *270*, 12250–12256.

(90) Wang, C.-I.; Yang, Q.; Craik, C. S. *Methods Enzymol.* **1996**, *267*, 52–68.
(91) Pasqualini, R.; Ruoslahti, E. *Nature* **1996**, *380*, 364–366.
(92) Smith, G. P.; Schultz, D. A.; Ladbury, J. E. *Gene* **1993**, *128*, 37–42.
(93) Wrighton, N. C.; Farrell, F. X.; Chang, R.; Kashyap, A. K.; Barbone, F. P.; Mulcahy, L. S.; Johnson, D. L.; Barrett, R. W.; Jolliffe, L. K.; Dower, W. J. *Science* **1996**, *273*, 458–463.
(94) Martens, C. L.; Cwirla, S. E.; Lee, R. Y.-W.; Whitehorn, E.; Chen, E. Y.-F.; Bakker, A.; Martin, E. L.; Wagstrom, C.; Gopalan, P.; Smith, C. W.; Tate, E.; Koller, K. J.; Schatz, P. J.; Dower, W. J.; Barrett, R. W. *J. Biol. Chem.* **1995**, *270*, 21129–21136.
(95) Yu, J.; Smith, G. *Methods Enzymol.* **1996**, *267*, 3–27.
(96) Roberts, B. L.; Markland, W.; Ley, A. C.; Kent, R. B.; White, D. W.; Guterman, S. K.; Ladner, R. C. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 2429–2433.
(97) Wells, J. A.; Lowman, H. B. *Curr. Opin. Biotechnol.* **1992**, *3*, 355–362.
(98) Dennis, M. S.; Lazarus, R. A. *J. Biol. Chem.* **1994**, *269*, 22129–22136.
(99) Martin, F.; Toniatti, C.; Salvati, A. L.; Ciliberto, G.; Cortese, R.; Sollazzo, M. *J. Mol. Biol.* **1996**, *255*, 86–97.
(100) Rickles, R. J.; Botfield, M. C.; Zhou, X. M.; Henry, P. A.; Brugge, J. S.; Zoller, M. J. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 10909–10913.
(101) Cadwell, R. C.; Joyce, G. F. *PCR Methods Applications* **1994**, *3*, S136–140.
(102) Cadwell, R. C.; Joyce, G. F. *PCR Methods Applications* **1992**, *2*, 28–33.
(103) Leung, D. W.; Chen, E.; Goeddel, D. V. *Technique* **1989**, *1*, 11–15.
(104) Cheadle, C.; Ivashchenko, Y.; South, V.; Searfoss, G. H.; French, S.; Howk, R.; Ricca, G. A.; Jaye, M. *J. Biol. Chem.* **1994**, *269*, 24034–24039.
(105) Luzzago, A.; Felici, F.; Tramontano, A.; Pessi, A.; Cortese, R. *Gene* **1993**, *128*, 51–57.
(106) Sun, Y.; Ball, W., Jr. *Biochim. Biophys. Acta* **1994**, *1207*, 236–248.
(107) Zhong, G.; Smith, G. P.; Berry, J.; Brunham, R. C. *J. Biol. Chem.* **1994**, *269*, 24183–24188.
(108) Koivunen, E.; Gay, D. A.; Ruoslahti, E. *J. Biol. Chem.* **1993**, *268*, 20205–20210.
(109) Koivunen, E.; Wang, B.; Ruoslahti, E. *J. Cell Biol.* **1994**, *124*, 373–380.
(110) Hoess, R. H.; Mack, A. J.; Walton, H.; Reilly, T. M. *J. Immunol.* **1994**, *153*, 724–729.
(111) De Ciechi, P. A.; Devine, C. S.; Lee, S. C.; Howard, S. C.; Olins, P. O.; Caparon, M. H. *Mol. Diversity* **1996**, *1*, 79–86.
(112) Lowman, H. B.; Bass, S. H.; Simpson, N.; Wells, J. A. *Biochemistry* **1991**, *30*, 10832–10838.
(113) Barbas, C. F.; Bain, J. D.; Hoekstra, D. M.; Lerner, R. A. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 4457–4461.
(114) Ku, J.; Schultz, P. G. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 6552–6556.
(115) Bianchi, E.; Folgori, A.; Wallace, A.; Nicotra, M.; Acali, S.; Phalipon, A.; Barbato, G.; Bazzo, R.; Cortese, R.; Felici, F.; et al. *J. Mol. Biol.* **1995**, *247*, 154–160.
(116) McConnell, S. J.; Hoess, R. H. *J. Mol. Biol.* **1995**, *250*, 460–470.
(117) O'Neil, K. T.; DeGrado, W. F.; Mousa, S. A.; Ramachandran, N.; Hoess, R. H. *Methods Enzymol.* **1994**, *245*, 370–386.
(118) Nord, K.; Nilsson, J.; Nilsson, B.; Uhlen, M.; Nygren, P. A. *Protein Eng.* **1995**, *8*, 601–608.
(119) Pessi, A.; Bianchi, E.; Crameri, A.; Venturini, S.; Tramontano, A.; Sollazzo, M. *Nature* **1993**, *362*, 367–369.
(120) Venturini, S.; Martin, F.; Sollazzo, M. *Protein Pept. Lett.* **1994**, *1*, 70–75.
(121) Tramontano, A.; Bianchi, E.; Venturini, S.; Martin, F.; Pessi, A.; Sollazzo, M. *J. Molecular Recognit.* **1994**, *7*, 9–24.
(122) Martin, F.; Toniatti, C.; Salvati, A. L.; Venturini, S.; Ciliberto, G.; Cortese, R.; Sollazzo, M. *EMBO J.* **1994**, *13*, 5303–5309.
(123) Adey, N. B.; Mataragnon, A. H.; Rider, J. E.; Carter, J. M.; Kay, B. K. *Gene* **1995**, *156*, 27–31.
(124) Bottger, V.; Stasiak, P. C.; Harrison, D. L.; Mellerick, D. M.; Lane, E. B. *Eur. J. Biochemistry* **1995**, *231*, 475–485.
(125) Chen, Y. C.; Delbrook, K.; Dealwis, C.; Mimms, L.; Mushahwar, I. K.; Mandecki, W. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 1997–2001.
(126) de la Cruz, V. F.; Lal, A. A.; McCutchan, T. F. *J. Biol. Chem.* **1988**, *263*, 4318–4322.
(127) Grihalde, N. D.; Chen, Y. C.; Golden, A.; Gubbins, E.; Mandecki, W. *Gene* **1995**, *166*, 187–195.
(128) Hoess, R.; Brinkmann, U.; Handel, T.; Pastan, I. *Gene* **1993**, *128*, 43–49.
(129) Hutchinson, A. M. *Mol. Biotechnology* **1995**, *3*, 47–54.
(130) Markland, W.; Roberts, B. L.; Saxena, M. J.; Guterman, S. K.; Ladner, R. C. *Gene* **1991**, *109*, 13–9.
(131) Parmley, S. F.; Smith, G. P. *Adv. Exp. Med. Biol.* **1989**, *251*, 215–218.
(132) Petersen, G.; Song, D.; Hugle-Dorr, B.; Oldenburg, I.; Bautz, E. K. *Molecular General Genetics* **1995**, *249*, 425–431.
(133) Stephen, C. W.; Lane, D. P. *J. Mol. Biol.* **1992**, *225*, 577–583.
(134) Stephen, C. W.; Helminen, P.; Lane, D. P. *J. Mol. Biol.* **1995**, *248*, 58–78.
(135) Geysen, H. M.; Rodda, S. J.; Mason, T. J.; Tribbick, G.; Schoofs, P. G. *J. Immunol. Methods* **1987**, *102*, 259–274.
(136) Wang, L. F.; Du Plessis, D. H.; White, J. R.; Hyatt, A. D.; Eaton, B. T. *J. Immunol. Methods* **1995**, *178*, 1–12.
(137) Rickles, R. J.; Botfield, M. C.; Weng, Z.; Taylor, J. A.; Green, O. M.; Brugge, J. S.; Zoller, M. J. *EMBO J.* **1994**, *13*, 5598–5604.
(138) Sparks, A. B.; Quilliam, L. A.; Thorn, J. M.; Der, C. J.; Kay, B. K. *J. Biol. Chem.* **1994**, *269*, 23853–23856.
(139) Sparks, A.; Rider, J.; Hoffman, N.; Fowlkes, D.; Quilliam, L.; Kay, B. *Proceedings of the National Academy of Sciences USA* **1996**, *93*, 1540–1544.
(140) Sparks, A. B.; Hoffman, N. G.; McConnell, S. J.; Fowlkes, D. M.; Kay, B. K. *Nature Biotechnology* **1996**, *14*, 741–744.
(141) Cwirla, S. E.; Peters, E. A.; Barrett, R. W.; Dower, W. J. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 6378–6382.
(142) Scott, J. K.; Smith, G. P. *Science* **1990**, *249*, 386–390.
(143) Balass, M.; Heldman, Y.; Cabilly, S.; Givol, D.; Katchalski-Katzir, E.; Fuchs, S. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 10638–10642.
(144) Yayon, A.; Aviezer, D.; Safran, M.; Gross, J. L.; Heldman, Y.; Cabilly, S.; Givol, D.; Katchalski-Katzir, E. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 10643–10647.
(145) Schumacher, T. N.; Mayr, L. M.; Minor, D. L., Jr.; Milhollen, M. A.; Burgess, M. W.; Kim, P. S. *Science* **1996**, *271*, 1854–1857.
(146) Liu, G.; Bryant, R. T.; Hilderman, R. H. *Biochemistry* **1996**, *35*, 197–201.
(147) Keller, P. M.; Arnold, B. A.; Shaw, A. R.; Tolman, R. L.; Van Middlesworth, F.; Bondy, S.; Rusiecki, V. K.; Koenig, S.; Zolla-Pazner, S.; Conard, P.; Emini, E. A.; Conley, A. J. *Virology* **1993**, *193*, 709–716.
(148) Schellekens, G. A.; Lasonder, E.; Feijlbrief, M.; Koedijk, D. G.; Drijfhout, J. W.; Scheffer, A. J.; Welling-Wester, S.; Welling, G. W. *Eur. J. Immunol.* **1994**, *24*, 3188–3193.
(149) Orlandi, R.; Menard, S.; Colnaghi, M. I.; Boyer, C. M.; Felici, F. *Eur. J. Immunol.* **1994**, *24*, 2868–2873.
(150) Galfre, G.; Monaci, P.; Nicosia, A.; Luzzago, A.; Felici, F.; Cortese, R. *Methods Enzymol.* **1996**, *267*, 109–115.
(151) Felici, F.; Luzzago, A.; Folgori, A.; Cortese, R. *Gene* **1993**, *128*, 21–27.
(152) Weber, P. C.; Pantoliano, M. W.; Thompson, L. D. *Biochemistry* **1992**, *31*, 9350–9354.
(153) Rebar, E. J.; Pabo, C. O. *Science* **1994**, *263*, 671–673.
(154) Rebar, E. J.; Greisman, H. A.; Pabo, C. O. *Methods Enzymol.* **1996**, *267*, 129–149.
(155) Wu, H.; Yang, W. P.; Barbas, C. F. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 344–348.
(156) Jamieson, A. C.; Kim, S. H.; Wells, J. A. *Biochemistry* **1994**, *33*, 5689–5695.
(157) Choo, Y.; Klug, A. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11163–11167.
(158) Choo, Y.; Sanchez-Garcia, I.; Klug, A. *Nature* **1994**, *372*, 642–645.
(159) Choo, Y.; Klug, A. *Curr. Opin. Biotechnol.* **1995**, *6*, 431–436.
(160) Krook, M.; Mosbach, K.; Lindbladh, C. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 849–854.
(161) Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. *Science* **1990**, *249*, 404–406.
(162) Christian, R. B.; Zuckermann, R. N.; Kerr, J. M.; Wang, L.; Malcolm, B. A. *J. Mol. Biol.* **1992**, *227*, 711–718.
(163) Hammer, J.; Takacs, B.; Sinigaglia, F. *J. Exp. Med.* **1992**, *176*, 1007–1013.
(164) McLafferty, M. A.; Kent, R. B.; Ladner, R. C.; Markland, W. *Gene* **1993**, *128*, 29–36.
(165) Kay, B. K.; Adey, N. B.; He, Y. S.; Manfredi, J. P.; Mataragnon, A. H.; Fowlkes, D. M. *Gene* **1993**, *128*, 59–65.
(166) Jellis, C. L.; Cradick, T. J.; Rennert, P.; Salinas, P.; Boyd, J.; Amirault, T.; Gray, G. S. *Gene* **1993**, *137*, 63–68.
(167) DeGraaf, M. E.; Miceli, R. M.; Mott, J. E.; Fischer, H. D. *Gene* **1993**, *128*, 13–17.
(168) Miceli, R. M.; DeGraaf, M. E.; Fischer, H. D. *J. Immunol. Methods* **1994**, *167*, 279–287.
(169) Takenaka, I. M.; Leung, S. M.; McAndrew, S. J.; Brown, J. P.; Hightower, L. E. *J. Biol. Chem.* **1995**, *270*, 19839–19844.
(170) Giebel, L. B.; Cass, R. T.; Milligan, D. L.; Young, D. C.; Arze, R.; Johnson, C. R. *Biochemistry* **1995**, *34*, 15430–15435.
(171) Bonnycastle, L. L.; Mehroke, J. S.; Rashed, M.; Gong, X.; Scott, J. K. *J. Mol. Biol.* **1996**, *258*, 748–762.
(172) Jacobsson, K.; Frykberg, L. *BioTechniques* **1995**, *18*, 878–885.
(173) Jacobsson, K.; Frykberg, L. *BioTechniques* **1996**, *20*, 1070–1081.
(174) Crameri, R.; Jaussi, R.; Menz, G.; Blaser, K. *Eur. J. Biochemistry* **1994**, *226*, 53–58.
(175) Crameri, R.; Blaser, K. *Int. Arch. Allergy Immunol.* **1996**, *110*, 41–45.
(176) van Zonneveld, A. J.; van den Berg, B. M.; van Meijer, M.; Pannekoek, H. *Gene* **1995**, *167*, 49–52.

(177) McCafferty, J.; Jackson, R. H.; Chiswell, D. J. *Protein Eng.* **1991**, *4*, 955−961.
(178) Light, J.; Lerner, R. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1073−1078.
(179) Maenaka, K.; Furuta, M.; Tsumoto, K.; Watanabe, K.; Ueda, Y.; Kumagai, I. *Biochem. Biophys. Res. Commun.* **1996**, *218*, 682−687.
(180) Corey, D. R.; Shiau, A. K.; Yang, Q.; Janowski, B. A.; Craik, C. S. *Gene* **1993**, *128*, 129−134.
(181) Eerola, R.; Saviranta, P.; Lilja, H.; Pettersson, K.; Lovgren, T.; Karp, M. *Biochem. Biophys. Res. Commun.* **1994**, *200*, 1346−1352.
(182) Soumillion, P.; Jespers, L.; Bouchet, M.; Marchand-Brynaert, J.; Sartiaux, P.; Fastrez, J. *Appl. Biochem. Biotechnol.* **1994**, *47*, 175−189.
(183) Vanwetswinkel, S.; Touillaux, R.; Fastrez, J.; Marchand-Brynaert, J. *Bioorg. Med. Chem.* **1995**, *3*, 907−915.
(184) Vanwetswinkel, S.; Marchand-Brynaert, J.; Fastrez, J. *Bioorg. Med. Chemistry Lett.* **1996**, *6*, 789−792.
(185) Widersten, M.; Mannervik, B. *J. Mol. Biol.* **1995**, *250*, 115−122.
(186) Ku, J.; Schultz, P. G. *Bioorg. Med. Chem.* **1994**, *2*, 1413−1415.
(187) Light, J.; Lerner, R. A. *Bioorg. Med. Chem.* **1995**, *3*, 955−967.
(188) Lowman, H. B.; Wells, J. A. *J. Mol. Biol.* **1993**, *234*, 564−578.
(189) Cunningham, B. C.; Lowe, D. G.; Li, B.; Bennett, B. D.; Wells, J. A. *EMBO J.* **1994**, *13*, 2508−2515.
(190) Li, B.; Tom, J. Y. K.; Oare, D.; Yen, R.; Fairbrother, W. J.; Wells, J. A.; Cunningham, B. C. *Science* **1995**, *270*, 1657−1660.
(191) Pannekoek, H.; van Meijer, M.; Schleef, R. R.; Loskutoff, D. J.; Barbas, C. D. *Gene* **1993**, *128*, 135−140.
(192) van Meijer, M.; Roelofs, Y.; Neels, J.; Horrevoets, A. J.; van Zonneveld, A. J.; Pannekoek, H. *J. Biol. Chem.* **1996**, *271*, 7423−7428.
(193) Tanaka, A. S.; Sampaio, C. A.; Fritz, H.; Auerswald, E. A. *Biochem. Biophys. Res. Commun.* **1995**, *214*, 389−395.
(194) Swimmer, C.; Lehar, S. M.; McCafferty, J.; Chiswell, D. J.; Blattler, W. A.; Guild, B. C. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 3756−3760.
(195) Scarselli, E.; Esposito, G.; Traboni, C. *FEBS Lett.* **1993**, *329*, 223−226.
(196) Djojonegoro, B. M.; Benedik, M. J.; Willson, R. C. *Bio/Technology* **1994**, *12*, 169−172.
(197) O'Neil, K. T.; Hoess, R. H.; Raleigh, D. P.; DeGrado, W. F. *Proteins* **1995**, *21*, 11−21.
(198) Gu, H.; Yi, Q.; Bray, S. T.; Riddle, D. S.; Shiau, A. K.; Baker, D. *Protein Sci.* **1995**, *4*, 1108−1117.
(199) Onda, T.; LaFace, D.; Baier, G.; Brunner, T.; Honma, N.; Mikāyāmā, T.; Altman, A.; Green, D. R. *Mol. Immunol.* **1995**, *32*, 1387−1397.
(200) Roberts, B. L.; Markland, W.; Ladner, R. C. *Methods Enzymol.* **1996**, *267*, 68−82.
(201) Laird-Offringa, I. A.; Balasco, J. G. *Methods Enzymol.* **1996**, *267*, 149−168.
(202) Gram, H.; Strittmatter, U.; Lorenz, M.; Gluck, D.; Zenke, G. *J. Immunol. Methods* **1993**, *161*, 169−176.
(203) Saggio, I.; Gloaguen, I.; Laufer, R. *Gene* **1995**, *152*, 35−39.
(204) Cabibbo, A.; Sporeno, E.; Toniatti, C.; Altamura, S.; Savino, R.; Paonessa, G.; Ciliberto, G. *Gene* **1995**, *167*, 41−47.
(205) Bottger, V.; Lane, E. B. *J. Mol. Biol.* **1994**, *235*, 61−67.
(206) du Plessis, D. H.; Wang, L. F.; Jordaan, F. A.; Eaton, B. T. *Virology* **1994**, *198*, 346−349.
(207) Yao, Z. J.; Kao, M. C.; Loh, K. C.; Chung, M. C. *FEMS Microbiol. Lett.* **1995**, *127*, 93−98.
(208) Morris, G. E.; Nguyen, C.; Nguyen, T. M. *Biochem. J.* **1995**, *309*, 355−359.
(209) South, V.; Searfoss, G. H.; French, S.; Cheadle, C.; Murray, E.; Howk, R.; Jaye, M.; Ricca, G. A. *Thromb. Haemostasis* **1995**, *73*, 144−150.
(210) Barchan, D.; Balass, M.; Souroujon, M. C.; Katchalski-Katzir, E.; Fuchs, S. *J. Immunol.* **1995**, *155*, 4264−4269.
(211) Burritt, J. B.; Quinn, M. T.; Jutila, M. A.; Bond, C. W.; Jesaitis, A. J. *J. Biol. Chem.* **1995**, *270*, 16974−16980.
(212) Bottger, V.; Bottger, A.; Lane, E. B.; Spruce, B. A. *J. Mol. Biol.* **1995**, *247*, 932−946.
(213) Roberts, D.; Guegler, K.; Winter, J. *Gene* **1993**, *128*, 67−69.
(214) Yao, Z. J.; Kao, M. C.; Chung, M. C. *J. Protein Chem.* **1995**, *14*, 161−166.
(215) Hammer, J.; Valsasnini, P.; Tolba, K.; Bolin, D.; Higelin, J.; Takacs, B.; Sinigaglia, F. *Cell* **1993**, *74*, 197−203.
(216) Sinigaglia, F.; Hammer, J. *Curr. Opin. Immunol.* **1994**, *6*, 52−56.
(217) Fujisao, S.; Matsushita, S.; Nishi, T.; Nishimura, Y. *Human Immunol.* **1996**, *45*, 131−136.
(218) Scott, J. K.; Loganathan, D.; Easley, R. B.; Gong, X.; Goldstein, I. J. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5398−5402.
(219) Daniels, D. A.; Lane, D. P. *J. Mol. Biol.* **1994**, *243*, 639−652.
(220) Castano, A. R.; Tangri, S.; Miller, J. E.; Holcombe, H. R.; Jackson, M. R.; Huse, W. D.; Kronenberg, M.; Peterson, P. A. *Science* **1995**, *269*, 223−226.
(221) Ding, L.; Coombs, G. S.; Strandberg, L.; Navre, M.; Corey, D. R.; Madison, E. L. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7627−7631.
(222) Krook, M.; Lindbladh, C.; Birnbaum, S.; Naess, H.; Eriksen, J. A.; Mosbach, K. *J. Chromatogr. A* **1995**, *711*, 119−128.
(223) Fang, R.; Qi, J.; Lu, Z. B.; Zhou, H.; Li, W.; Shen, J. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 53−56.
(224) Saggio, I.; Laufer, R. *Biochem. J.* **1993**, *293*, 613−616.

CR960065D